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CONNECTIVE TISSUE REACTIONS IN NORMAL AND IMMUNIZED MICE TO A RETICULOTROPIC STRAIN OF *TRYPANOSOMA CRUZI*

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The large amount of work on the general pathology and histopathology of Chagas' disease in man has been summarized by Chagas et al (1929) and Torres (1941). Detailed studies, especially of heart lesions, have been made by many authors, for example, by Mazza et al (1939) and Torres and Duarte (1948). In animals, special mention should be made of the reports by Mazza and Jörg (1935a and 1943) and Wood (1937). Wood made an excellent study of *Trypanosoma cruzi* in the blood and reticular tissues of a small series of *Peromyscus californicus*, the white-footed mouse.

The early investigators (see Chagas et al, 1929) emphasized that the leishmaniform stages of *Trypanosoma cruzi* have a predilection for (1) heart and skeletal muscle and (2) reticulo-endothelial, glial and fat cells. Other authors, notably de Souza Campos (1929), Dias (1932 and 1934) and Mazza and his associates (1935a and b and 1943), emphasized the importance of macrophages as host cells for the infection. These authors, in general, studied terminal stages of the infection.

More recently, it has been found that strains of parasites differ in their tissue tropism and that the genetic constitution of the host plays a major role in the virulence of the parasite. In an experimental study of various strains of the parasite in guinea pigs and dogs, Badínez (1945) found some that were chiefly myotropic and others that were chiefly reticulotropic. In the same year, Pizzi found that the myotropic strain only rarely parasitized reticulo-endothelial cells but localized largely in the heart, skeletal muscle, smooth muscle of the intestines and adipose tissue. Both Hauschka and Pizzi et al emphasized the importance of the genetic constitution of the host. Hauschka (1949) showed clearly that by using highly homozygous male mice of inbred stock, two strains of *T. cruzi* exhibited well-defined and constant differences in virulence, degree of affinity for certain host tissues, susceptibility to Bayer 7602, and response to environmental temperature. One of his strains was probably similar to the strain used in the present study. Pizzi et al (1948–1949 and 1949) found that the reticulotropic strain used in the present study exhibited remarkable and constant differences in virulence when inoculated into 8 different standardized inbred strains of mice. Pizzi and Prager (1953) found further that the highest virulence was exhibited in C_3H mice and that the infection could be standardized by continuous passage in them.

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* Formerly the Department of Bacteriology and Parasitology.

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In the present investigation, particular attention is paid to the elective location of the reticulotropic strain of *T. cruzi* within different connective tissue cells and the general connective tissue reactions of C_3H mice to the infection. The authors believe the work is significant because it is, as far as they know, the first detailed study of the connective tissue reactions to a reticulotropic strain of *T. cruzi* in a genetically standardized host. It indicates the early systemic invasion of the fixed macrophages by this strain as contrasted to the minor invasion of these cells by the myotropic strains. It shows the intense but essentially non-effective reactions of the lymphoid-macrophage system* in nonimmunized mice as compared to the milder but generally effective reactions in immunized animals. Finally, it shows the differential phagocytic activity of connective tissue cells against highly virulent blood stages and low virulent culture forms in the nonimmunized host and the enhanced activity of macrophages, especially inflammatory macrophages, against virulent parasites in the immunized host.

Our terminology for the connective tissues cells is given by Taliaferro and Bloom (1945) and Taliaferro (1949). We have designated as inflammatory macrophages those large free activated phagocytes which have mobilized directly from fixed reticular, littoral or adventitial cells or have developed heteroplastically from agranulocytes. We have designated as medium or intermediate polyblasts, the inflammatory macrophages intermediate in size and nuclear structure between agranulocytes and large macrophages, which have lost their typical lymphoid or monocytic characteristics.

MATERIALS AND METHODS

A highly inbred strain of C_3H mice (see Law, 1948, and Committee etc., 1952) of mice was used exclusively and was originally obtained from Jackson Memorial Laboratories and has been maintained by inbreeding and genetic control in the Department of Parasitology of the University of Chile since 1946. Equal numbers of about 2-month old males and females were ordinarily used (table 1). The dogs were 6-week old puppies.

The strain of *T. cruzi* was isolated from *Triatoma infestans* found in the northern part of Chile in 1945 and was maintained until 1950 by passage through dogs, guinea pigs and mice. In 1950, it showed an irregular virulence and a marked tendency to develop in reticular cells of the reticular tissues, littoral cells, fibroblasts and histiocytes of the loose connective tissue and fat cells. The invasion of muscle fibers (including the heart) was comparatively rare (Badínez, 1945). From 1950 on, the strain has been maintained by regular serial transfer every 10 days in C_3H mice with the result that the virulence of the parasite has been stabilized and the infection standardized (Pizzi and Prager, 1953). It has also been maintained in culture for 7 years by serial transfer every 10 days in Senekjie's (1943) medium. These culture forms show a marked diminution in virulence and have been used for immunization (see below). As shown in table 1, series of mice were infected and challenged once with virulent trypanosomes suspended in 0.85% sodium chloride and were immunized once with low virulent culture forms, mainly crithidial and leishmaniform parasites. Subcutaneous injections were made laterally on the midback in a shaved area.

Parasitemias were obtained from daily parasite counts by the following method (Pizzi et al, 1949; Jarpa et al, 1950, and Pizzi and Prager, 1953): A measured quantity of blood (5 μl) was obtained from the tail with a specially marked hemoglobin pipette, was put on a slide and covered with a plastic cover slip measuring 22×22 mm which had been ruled in mm. The parasites were then counted on a whole or a part of the slide depending upon their number and are given in text figure 1 per μl blood.

Various tissues were taken from the following three types of infections: (1) Six series of nonimmune mice were infected with virulent blood trypanosomes (virulent infection). (2) Three series of mice were immunized with low virulent

* A combination of the reticulo-endothelial system of Aschoff and the lymphoid system, which includes, among other cells, all macrophages and nongranular leucocytes and cells intermediate between them (Taliaferro and Mulligan, 1937, and Taliaferro, 1949).

TABLE 1.—*Data* on 215 mice and 6 dogs infected with* T. cruzi. *Two or more normal mice were always included in each series.*

Series*	Number of mice	Age in weeks	First injection			Days between 1st and 2nd injection	Challenge injection			Used in		
			Type of parasite	Route	Millions of parasites per mouse		Type of parasite	Route	Millions of parasites per mouse	Plate	Figure	Text figure
1	20	7	Vir.†	IP	0.15					1	4	
										1	5	
										3	2	
										4	1	
										4	4	
										5	3	
2	20	8	Vir.	IP	0.15					1	2	
										3	1	
										4	2	
										5	1	
										5	4	
3	10	8	Vir.	IP	1.0							
4	10	10	Vir.	IP	0.06					3	3	
5	40	8	Culture‡	IP	3.5	23	Vir.	IP	0.75			
6	40	8	Culture	SC	150.0	24	Vir.	SC	2.0	1	3	1
										2	1–4, 6, 7	
										5	2	
7	15	10	Vir.	SC	2.0							
8	20	8	Vir.	SC	1.1							2
9	40	11.5	Culture	SC	180.0	22	Vir.	SC	3.2	2	5, 8	3
	Dogs											
10	6	6	Vir.	IP	2.0					1	1	
										4	3	

* For embedding and staining methods, see *Materials and Methods.*
† Virulent blood trypaniform parasites.
‡ Low virulent culture forms.

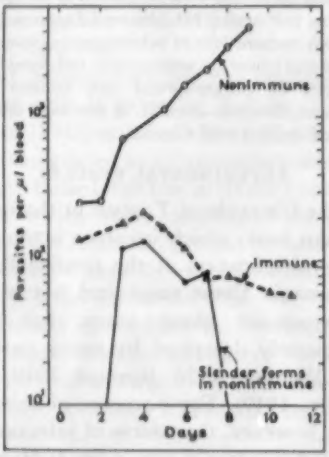
trypanosomes (immunizing infection). (3) Three series of mice immunized as in (2) were challenged with virulent blood trypanosomes (immunized and challenged mice). In the series in section 1, one or two mice were killed every day for 10 days (or as long as mice lived). In the series in section 2, one mouse was killed every other day through the 22nd to 24th day. In the series in section 3, one mouse was killed every day for 10 days, every other day for 2 weeks and every 3rd or 4th day for a month longer. In addition, in each section, extra mice were killed at earlier intervals. Two or more normal mice were killed in each series. For comparative purposes, tissues from 6 dogs were studied. Dogs usually died in 30 to 35 days. Occasionally one recovered. Thus, the infection in them was less acute than in mice. The reader is referred to table 1 for various details in regard to these series.

The following tissues were prepared for study from freshly sacrificed animals: skin, spleen, right inguinal and lumbar-aortic lymph nodes, bone marrow, thymus, adrenals, liver, skeletal muscle, heart, lungs and pieces of the small and the large intestine. These will be considered in succession under experimental results. They were immediately fixed from 4 to 6 hours in Zenker-formol (9 parts of Zenker's solution without acetic acid to 1 part of neutral formalin). Series 1 through 5 and 10 were embedded in celloidin and, after sectioning, were stained with hematoxylin-eosin-azure II, according to Maximow (see Buchsbaum and Loosli, 1936). In series 6 through 9, the same staining method was used after a combined embedding technique with celloidin and paraffin according to Péterfi (1921, also see Langeron, 1949). Stretch preparations of subcutaneous connective tissue and omentum were usually wet-fixed for 15 minutes in Zenker-formol and stained with hematoxylin-eosin-azure II. A few were dried in air and stained with Giemsa.

EXPERIMENTAL RESULTS

The life cycle of *T. cruzi* in the mammalian host, which involves intracellular multiplication of the nonflagellated leishmania tissue stage and not of the trypanosome blood stage, has been adequately described by many authors (see Wenyon, 1926; Brumpt, 1949; and Hoare, 1949). For purposes of orientation, however, the course of infection of the reticulotropic strain in C_3H mice will be briefly described as reported by Pizzi and Prager (1953). Following the injection of 150,000 or more virulent trypanosomes subcutaneously, trypano-

somes rapidly infected host cells of the local area and transformed into leishmania stages which divided by binary fission approximately every 12 hours for about 3 days. Then, most parasites retransformed into trypanosome stages and invaded the blood stream. Since such forms reinfected host cells in various tissues at irregular intervals to reinitiate multiplicative leishmaniform stages, reproduction ceased to be synchronous. The parasitemia increased in intensity from the 3rd day to a peak on the 9th to 11th day, at which time most of the mice died. (A few lived to 15 days.) It is interesting that slender trypanosomes were numerous at the start of the parasitemia, but steadily decreased thereafter (text fig. 1, nonimmune). When only about 60,000 virulent trypanosomes were injected (series 4), the course of the infection was essentially similar, but an occasional mouse lingered on until the 23rd day. Low grade infections which were usually nonlethal followed the injection of culture forms into normal mice (Pizzi and Prager, 1952) or of virulent trypanosomes into mice immunized 21 to 24 days (text fig. 1, immune).



TEXT FIG. 1.—Mean parasitemia in nonimmune and immune mice and the changes in number of slender forms in the nonimmune group (series 6, see table 1 for details).

Loose connective tissue: skin

Two types of skin preparations were made. The first one involved sections of skin from the dorsolateral area of mice inoculated by the intraperitoneal route. The second one involved stretch preparations of the subcutaneous loose connective tissue in the area subcutaneously injected.

Virulent infection.—The sections from intraperitoneally infected animals were studied chiefly to ascertain the intracellular localizations of the leishmania. A few parasites were first found on the 5th day in macrophages, fat cells and fibroblasts of both the derma and hypodermis. They increased in number. From the 9th to the 15th day (the last day on which any animals survived in this particular series), numerous inflammatory foci were scattered throughout the derma and hypodermis. Within these areas, parasites were found in medium polyblasts in addition to the cells previously mentioned (pl. 1, fig. 2 and cf. also pl. 1, fig. 1 from a dog). It is interesting to note how wet and dry fixation in the stretch preparations affected cells (cf. pl. 1, fig. 1 with pl. 5, fig. 4).

In stretch preparations at or near the site of subcutaneous inoculations, a few parasites were found on the 1st day. They rapidly increased in number and were numerous by the 3rd day. They remained at this high level throughout the rest of the infection (9 days was the longest survival time in this series). Mild inflammatory foci appeared on the 3rd and 4th days and continued to be present as in the animals inoculated intraperitoneally. The majority of the leish-

maniform stages found in host cells were normal in appearance. Only occasional ones appeared damaged within hematogenous macrophages in these initial infections.

Immunizing infection.—Stretch preparations were studied at early intervals after the subcutaneous injection of low virulent culture forms. At 6 hours, free crithidial forms occurred in large numbers in the subcutaneous tissue and were being actively phagocytosed and destroyed by local macrophages. The crithidia apparently became leishmaniform shortly after injection. Inflammatory reactions were more intense than in the preceding group, and more heterophils were present during the first few hours. Occasional heterophils contained one or two parasites. In contrast, virulent parasites were practically never seen in heterophils. At 14 hours and thereafter, hematogenous macrophages became increasingly important in removing parasites. Intense phagocytosis occurred at 24 hours. Thereafter, only a few parasites were found in the local area or elsewhere in the skin.

Immunized and challenged mice.—Stretch preparations of the subcutaneous loose connective tissue at or near the site of the challenging inoculation were especially valuable for comparing this group with the preceding groups. In marked contrast to fatal infections, virulent parasites, although numerous on the 1st day after inoculation, decreased markedly for 2 days and stayed at this low level for 9 days. Most of them were destroyed in inflammatory macrophages in the first several hours. With few exceptions, that were probably due to variability in the host response, intense inflammatory foci were continuously present from the 1st day. Thus, in the immune as compared to the nonimmune mice, parasites were more rapidly destroyed and inflammation was more intense.

Spleen

Normal mice.—The histology of the mouse spleen has been adequately described by Murray (1948), Snook (1950) and Singer (1954). In our material, the lymphatic nodules occurred at the periphery of the dense lymphatic tissue of the periarterial sheaths of white pulp. They were arranged in general as were the nodules in the cortex of the lymph nodes. The periarterial sheaths were surrounded by what Snook and others term the marginal zone and what we, following Taliaferro and Cannon (1936) and Taliaferro and Mulligan (1937), term the transitional zone. The nodule, when located next to the transitional zone, was separated from it by cleft-like spaces which Snook terms the perifollicular spaces. Snook reported that the nodular arteries empty directly into the perifollicular spaces. Singer described the perifollicular spaces as a series of anastomosing cleft-like spaces which were lined by cells closely resembling the littoral cells of the venous sinuses. He believes they may actually be venous sinuses. Knisely (1936) described a closed vascular system between the arterial capillaries and either the venules or venous sinuses. It should be further noted that Snook classified the mouse spleen as nonsinusal or as having primordial veins in the red pulp rather than true sinuses.

In uninfected animals, the lymphatic nodule was composed chiefly of small lymphocytes and generally contained a small germinal center in which varying numbers of large and medium lymphocytes were undergoing mitosis. In addition, there were many transitional stages between reticular cells and large basophil lymphocytes. We agree with

Legends for Plates

All drawings and photomicrographs are from tissues from C_3H mice, either normal or infected with *T. cruzi*, except for figure 1 in plate 1 and figure 3 in plate 4, which are from a dog. All drawings were made with the aid of a camera lucida. For injection data, see table 1, and for the histological techniques, see *Materials and Methods*.

PLATE 1.—Drawings from the skin and spleen. ×1500.

FIG. 1.—Infected macrophages and fibroblasts in a stretch preparation of the subcutaneous fat tissue from a 25-day infection with virulent parasites (series 10 from a dog).

FIG. 2.—Infected fibroblasts in the derma from a 13-day infection with virulent parasites (series 2).

FIG. 3.—Red pulp of the spleen from a 6-day infection with virulent parasites (series 6). Plasma cells are arising from the basophil precursors of red cells.

FIG. 4.—Infected macrophages in the red pulp of a spleen from a 7-day infection with virulent parasites (series 1).

FIG. 5.—Portion of a lymphocytopoietic center of a splenic nodule in a normal mouse (series 1) showing various stages (2 to 5) in the transformation of a typical reticular cell (1) into a large basophil lymphocyte (6).

PLATE 2.—Photomicrographs of spleens. ×24.

FIG. 1.—Uninfected mouse (series 6).

FIG. 2, 3 and 4.—Mice infected 4, 9 or 10 days previously with virulent blood forms (series 6).

FIG. 5.—Immunized mouse infected 20 days previously with low virulent culture forms (series 9).

FIG. 6 and 7.—Immune mice challenged 6 or 10 days previously with virulent trypanosomes (series 6).

FIG. 8.—Immune mouse challenged 11 days with virulent blood forms (series 9). Note more lymphoid hyperplasia and much less myelopoiesis than in figure 7.

PLATE 3.—Drawings from peripheral lymph nodes. ×1500.

FIG. 1.—Medullary and cortical areas in a peripheral lymph node from a 14-day infection with virulent parasites (series 2). The cortex contains areas of erythropoiesis. Note the cyst-like macrophages.

FIG. 2.—A portion of the medulla of a peripheral lymph node from a 7-day infection with virulent parasites (series 1). Note the presence of monocytoid lymphocytes (M) transitional in structure between lymphocytes and monocytes, and of medium-sized polyblasts (M Pbl). The macrophages with the peculiar nuclei (Mac) may be early stages in the formation of the macrophages in figures 1 and 3 of this plate.

FIG. 3.—A portion of the medulla of a peripheral lymph node from a 23-day infection with virulent parasites (series 4).

PLATE 4.—Drawings from the thymus, cartilage and bone. ×1500.

FIG. 1.—Reticular cells in the cortex of the thymus from a 7-day infection with virulent parasites (series 1). Note that some cells contain only parasites and others only cellular debris.

FIG. 2.—Cartilage from a 7-day infection with virulent parasites (series 2). Note the chondrocyte infected with leishmaniform *T. cruzi*.

FIG. 3.—A portion of the cortex of the thymus from a 7-day infection with virulent parasites (series 10 from a dog). Note the infected reticular cell and the exposure of reticular cells due to the loss of lymphocytes (thymocytes).

FIG. 4.—Bone from a 7-day infection with virulent parasites (series 1). Note the osteocyte infected with leishmaniform *T. cruzi*.

PLATE 5.—Drawings from the liver, intestine and omentum. ×1500.

FIG. 1.—A Kupffer cell in the liver packed with normal-appearing leishmaniform parasites. From an 11-day infection with virulent parasites (series 2).

FIG. 2.—A small inflammatory focus in the liver from a 9-day infection with virulent parasites (series 6). Inflammatory macrophages are clearing up parasites and debris probably caused by the rupture of a cell similar to that in the preceding figure. Note that some of the parasites, especially in the upper left, appear damaged.

FIG. 3.—An infected adventitial cell in the intestine from a 4-day infection with virulent parasites (series 1).

FIG. 4.—An infected macrophage in an omental stretch preparation dried in air and stained with Giemsa (series 2). Compare its nuclear structure with the wet-fixed specimens in plate 1, figure 1.

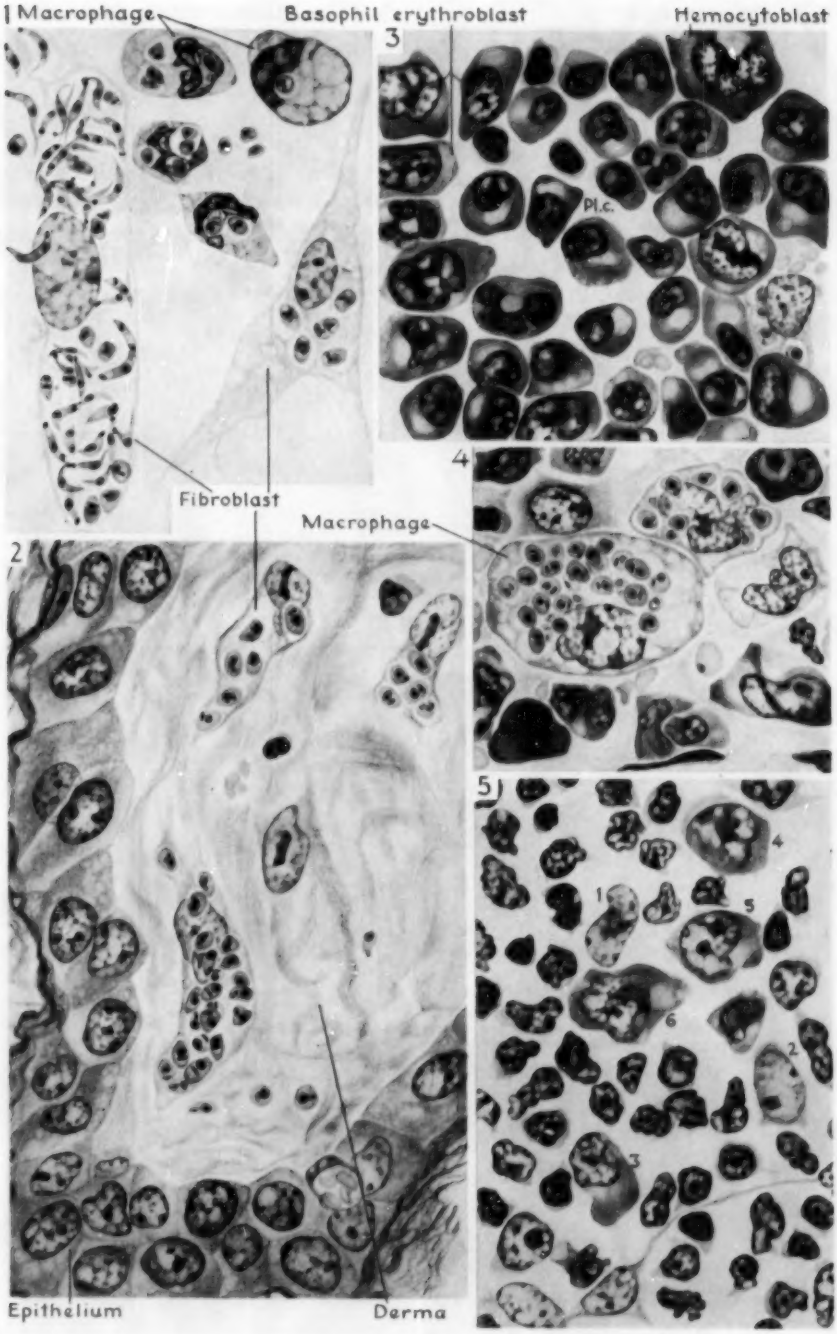

1
Macrophage
Basophil erythroblast
Hemocytoblast
3
Pl.c.
4
Fibroblast
Macrophage
2
5
Epithelium
Derma

Plate 1

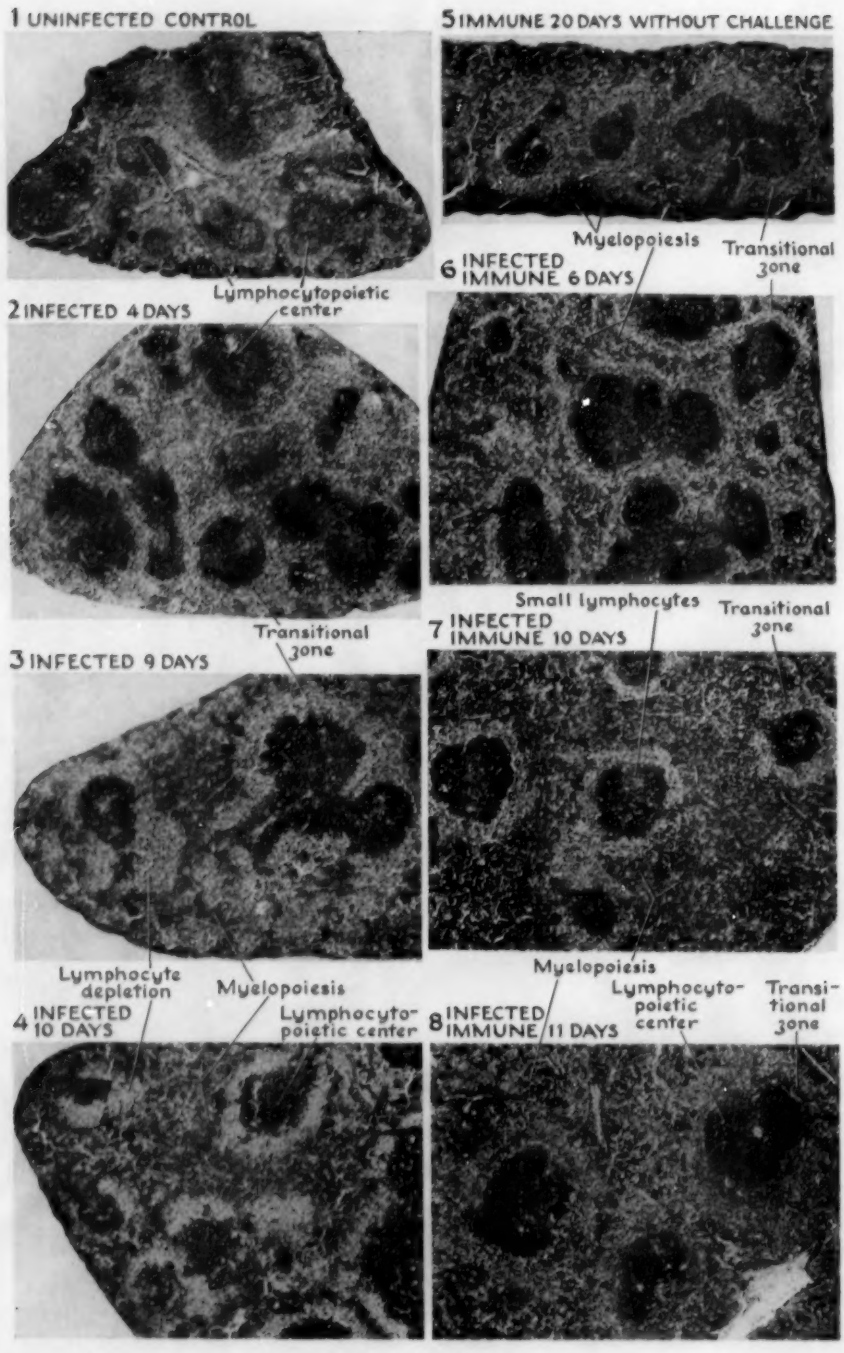

1 UNINFECTED CONTROL
5 IMMUNE 20 DAYS WITHOUT CHALLENGE
Myelopoiesis
Transitional zone
6 INFECTED IMMUNE 6 DAYS
Lymphocytopoietic center
2 INFECTED 4 DAYS
Small lymphocytes
Transitional zone
7 INFECTED IMMUNE 10 DAYS
Transitional zone
3 INFECTED 9 DAYS
Myelopoiesis
Lymphocyte depletion
Myelopoiesis
Lymphocyto-poietic center
Transi-tional zone
4 INFECTED 10 DAYS
8 INFECTED IMMUNE 11 DAYS

PLATE 2

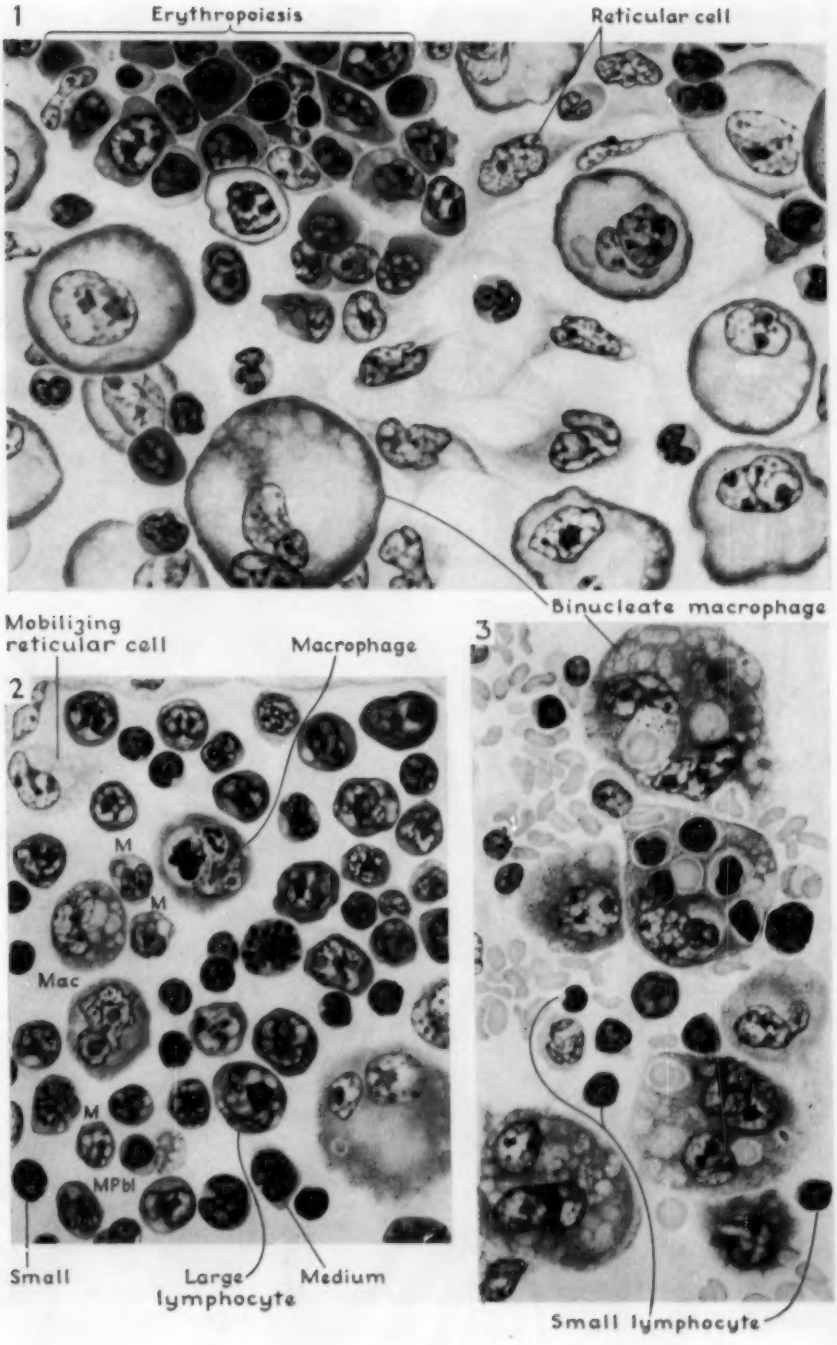

1
Erythropoiesis
Reticular cell
Binucleate macrophage
2
Mobilizing reticular cell
Macrophage
M
M
Mac
M
MPbl
Small
Large lymphocyte
Medium
3
Small lymphocyte

Plate 3

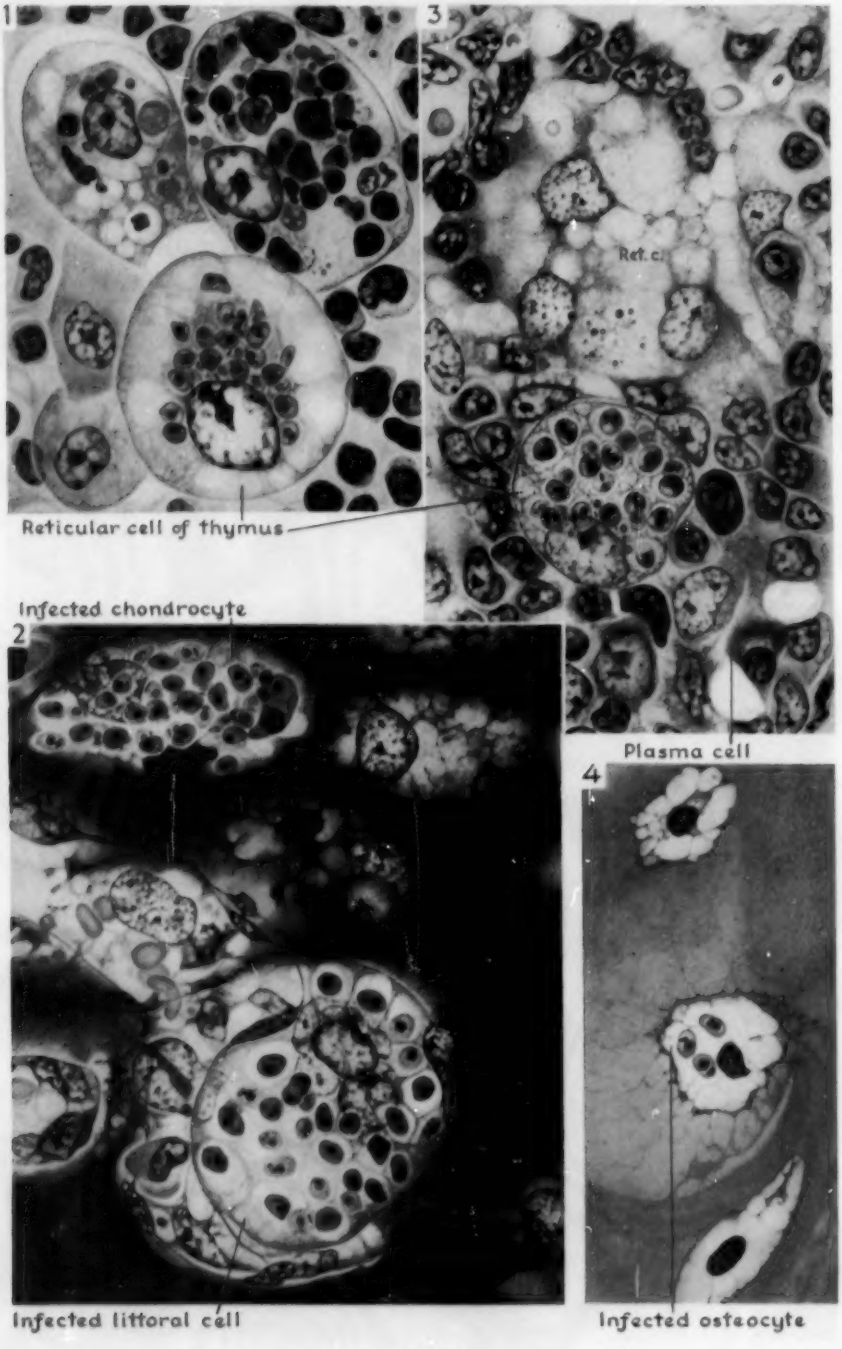

1
Reticular cell of thymus
3
Ret. c.
Plasma cell
Infected chondrocyte
2
Infected littoral cell
4
Infected osteocyte

PLATE 4

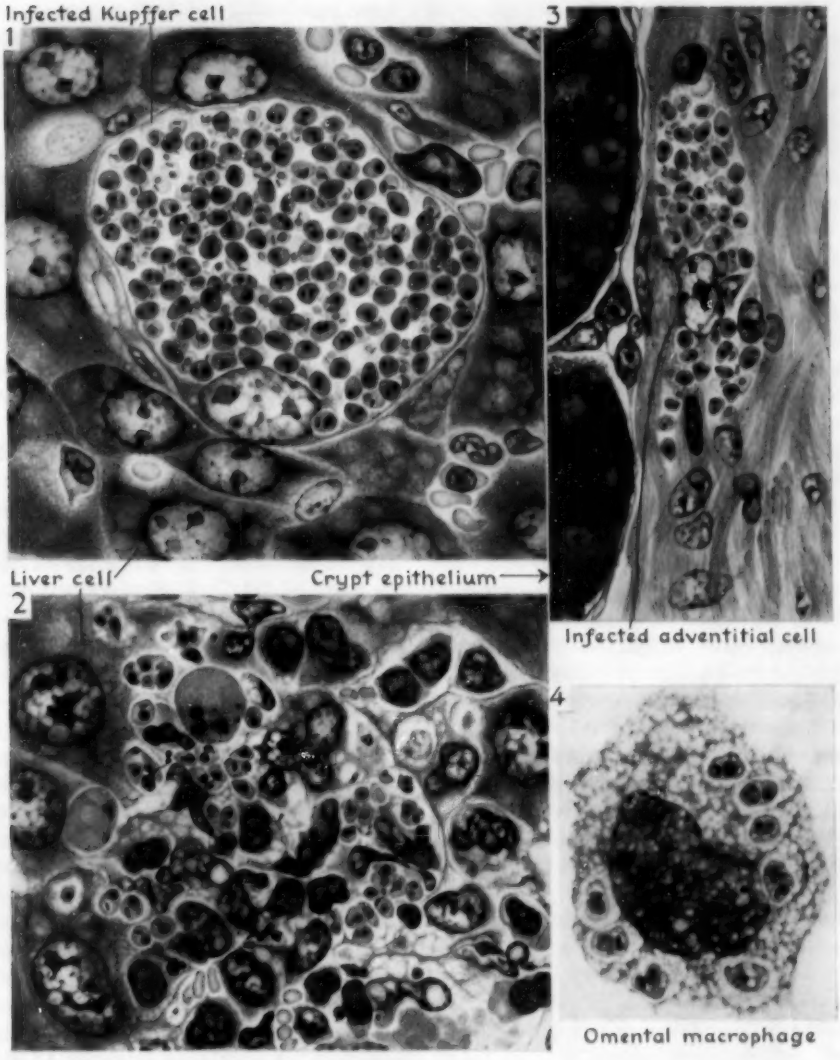


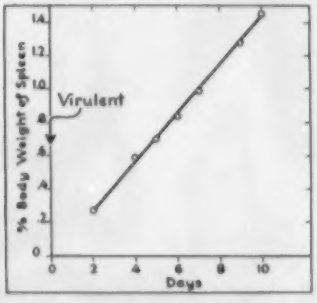
Plate 5

the generally accepted interpretation that these transitional stages represent the mobilization of reticular cells and their transformation into large lymphocytes (pl. 1, fig. 5). The lymphocytes resulting from this transformation were generally smaller and less basophil in normal than in infected mice. The lymphocytopoietic area was poorly defined. Under low magnifications, however, it could be distinguished from the rest of the nodule by its slightly lighter staining in normal animals (pl. 2, fig. 1) and by its darker staining due to the presence of large lymphocytes in infected animals (pl. 2, fig. 8).

The transitional zone consisted mainly of fixed reticular cells with varying numbers of free cells. These were almost entirely medium lymphocytes. In infected mice, the free cells were sometimes a mixture of small and medium lymphocytes and at times almost exclusively erythrocytes.

The red pulp proper contained numerous small active foci of myelopoiesis in which could be found the entire red cell series from large hemocytoblasts to normoblasts. Developing megakaryocytes were also a prominent feature of the myelopoietic areas (see description by Singer, 1954). Although granulopoiesis also occurred, it was generally localized to sites next to the trabeculae or capsule and was normally restricted in amount.

Virulent infection.—Splenomegaly developed as the infection progressed. Thus the mean weight of the spleen in percent body weight of 5 uninfected normal mice was $0.29 \pm 0.03\%$ whereas, in experiment 8, after the injection of 1.1 million trypanosomes subcutaneously, the relative weight of the spleen increased until it reached 1.45% of the body weight on the 10th day, which was the longest period any mouse survived (text fig. 2). In experiment 7, in which mice received 2 million parasites subcutaneously, the increase in spleen weight was more irregular but reached 1.3% of the body weight on the 9th day. Thus, the relative increase of the spleen in these 2 series was 5- and 4.6-fold, respectively. These values are more than 3 times the enlargement reported for rats infected with *Bartonella muris* by Cannon and McClelland (1929), but are smaller than those in CFI mice infected with *Plasmodium berghei* as judged by the cross sections published by Singer (1954). In immunized and challenged mice (text fig. 3), the enlargement more nearly approached Singer's findings.



TEXT FIG. 2.—Changes in spleen size expressed in percent body weight in nonimmune mice (series 8, see table 1 for details). Each data point is from one mouse.

Parasites were first seen on the 4th day, especially in reticular cells and free macrophages. They began to be seen in inflammatory macrophages on the 6th day. In general, they were normal in appearance, but a relatively small proportion appeared damaged. They were never found in lymphocytes or myeloid stem cells.

By the second day or shortly thereafter, hyperplastic and degenerative changes proceeded concomitantly. The hyperplastic aspects predominated at first and the degenerative ones later. In some animals as early as the second day, a depletion of lymphocytes at the center of the nodules gave the appearance of a reaction center surrounded by a peripheral layer of predominantly small lymphocytes. In other animals, enlarged ill-defined lymphocytopoietic centers contained free cells, predominantly large lymphocytes. These changes were associated with the mobilization of a large number of reticular cells into large basophil lymphocytes. The mobilization was at first noted in the nodules and later in other white pulp, red pulp and the transitional zone (pl. 2, fig. 2). For several days, there were only a few mitoses, chiefly of reticular cells. Soon thereafter and

invariably later in the infection, many large and medium lymphocytes also divided mitotically throughout the spleen but chiefly in the nodules. The lymphocyte population of the spleen contained an increasing proportion of large lymphocytes. Before their pronounced destruction, lymphocytes migrated from the nodules into the transitional zone and into the red pulp.

By the 4th day, many inflammatory-like foci occurred in the red pulp and frequently in the areas of myelopoiesis. These consisted of one to several rounded macrophages or outstretched reticular cells filled with parasites encircled by a collection of smaller polyblasts, intermediate in nuclear structure between agranulocytes and large macrophages, and a few lymphoid cells (pl. 1, fig. 4 and cf. a somewhat similar focus in the liver, pl. 5, fig. 2).

By the 6th day, the total size of the spleen had markedly increased and concomitantly there was a pronounced macrophage and lymphoid hyperplasia. The increase in spleen size was correlated with the production of new white pulp. The enlarged periarterial sheaths extended into the frequently enlarged transitional zone. Along with the production of new white pulp, there was a hyperplasia of the myeloid centers scattered throughout the red pulp. In later stages, myeloid tissue, mostly erythropoietic in nature, developed along the trabeculae and largely filled the red pulp. In general, it did not extend into the transitional zone (pl. 2, fig. 4), but in a few animals it invaded the periphery of the regressing nodules.

The mobilization of reticular cells and their transformation into large basophil lymphocytes was extensive on the 6th day although somewhat less than on the 4th day. In addition, a continuous series of cells could be found ranging in size from the medium lymphocyte to the large macrophage. Nuclear changes accompanied this hypertrophy of agranulocytes into macrophages. These findings indicated an extensive heteroplastic development of macrophages from agranulocytes as well as a direct mobilization of reticular cells into macrophages.

Degenerative processes were marked by the 8th day. Many areas in the periarterial sheaths and nodules were depleted of lymphocytes, and marked lymphorrhexis was present in both the white and the red pulp. In the myelopoietic areas in the red pulp, the smaller cells of the erythropoietic series were frequently degenerate in appearance and, among the normally appearing cells, plasma cells predominated. The developing megakaryocytes possessed pycnotic nuclei or were obviously degenerate. Throughout the spleen, macrophages were filled with cellular debris and parasites (see below). In spite of this extensive cellular destruction, there were some mitoses in the reticular cells and many mitoses in the larger lymphocytes of the white pulp and in the hemocytoblasts in the myelopoietic centers of the red pulp.

Much of the interplay of lymphocytopoiesis, the migration of lymphocytes into the red pulp, and the destruction of lymphocytes was reflected in the transitional zone. Early in the infection many small lymphocytes appeared in this zone as they disappeared from the nodules, but as the infection progressed the ratio of larger lymphocytes increased. At the height of lymphoid hyperplasia, the transitional zone was greatly enlarged and contained predominately large lymphocytes as did the white pulp in general (pl. 2, fig. 2). During the latter part of the infection, when destructive processes predominated, the transitional zone lost most of its lymphocytes but its increased ex-

tent was clearly seen as reticulum depleted of lymphocytes (pl. 2, fig. 3 and 4).

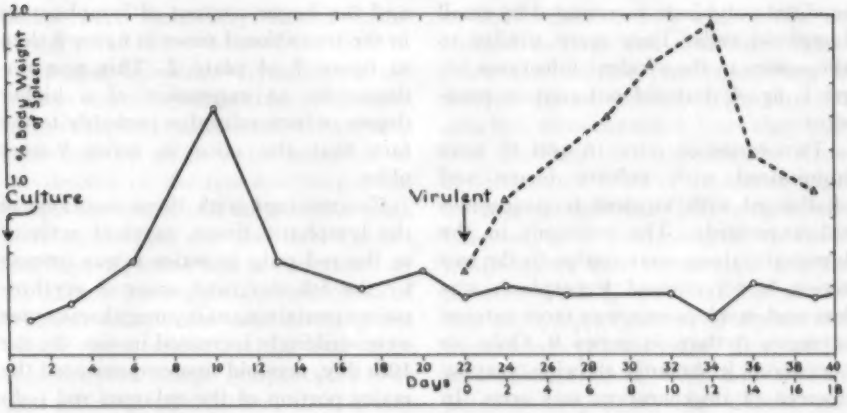
A large number of leishmaniform parasites, both extracellular and within macrophages, occurred in the periarterial sheaths and especially in the reticular cells and free macrophages of the marginal zone, the red pulp and the myeloid foci. Many reticular cells lining the venous sinuses were largely filled with parasites and, although swollen, retained their outstretched endothelial-like position. It is of interest that the cells lining the splenic veins behaved similarly. If these cells actively phagocytose parasites, the process is similar to that previously described by Taliaferro and Mulligan (1937) in the small splenic veins of monkeys infected with malaria. Many of the macrophages engorged with cellular debris and parasites were degenerating. In general, their appearance was similar to that seen in the later stages of overwhelming infections of *Plasmodium gallinaceum* in chickens, as described by Taliaferro (unpublished work). Although most parasites were normal in appearance, a few, especially in the free macrophages during late stages in the infection, were obviously degenerate as indicated by one or more of the following: a swollen abnormally stained nucleus or parabasal body, a fragmented nucleus, and/or thickened layers of protoplasm at the cell periphery.

By the 9th day, lymphocytes were so reduced in number that areas of almost lymphocyte-free reticulum were apparent throughout the white pulp including the nodules. The perifollicular spaces, enlarged transitional zones and red pulp were also largely free of lymphocytes (pl. 2, fig. 3). Both the transitional zones and the red pulp were markedly hyperemic at this time. Myelopoietic areas were partially depleted of lymphoid cells. Plasma cells were strikingly increased in the red pulp and were apparently arising in part from various basophil precursors of cells in the myeloid areas (pl. 1, fig. 3). All of the megakaryocytes were apparently degenerating. In spite of these changes, less debris and actual degeneration were apparent than on the 8th day.

During the terminal stages of the infection, leishmaniform parasites greatly increased in number in all areas. They were usually found in littoral cells, reticular cells, free macrophages, and polyblasts transitional between agranulocytes and macrophages. Occasional ones were seen in degenerating megakaryocytes. None was seen in lymphocytes, erythrocytes or heterophils. At times, atypical macrophages were encountered (see especially cell marked Mac in a lymph node in pl. 3, fig. 2). They are probably the forerunners of the peculiar cyst-like giant cells first described by Torres and de Azevedo (1929a and b; see also pl. 3, fig. 1 and 3).

Immunizing infection.—In the mice with a mean body weight of 22 g which were inoculated with 180 million culture forms, the spleen increased in size for about 10 days and then regressed until it was only slightly larger than normal on the 22nd day (text fig. 3). The peak size was almost identical to that reached on the same day in mice with fatal infections (text fig. 2). The only difference was that the rise of relative spleen weight was sigmoid rather than linear in the nonfatal infections when arithmetic plots of spleen weight in percent body weight were made against time (cf. text fig. 2 and 3).

A histological study of the immunized mice revealed all the various processes described for the virulent infection. The changes, however, largely involved hyperplasia of the lymphatic



TEXT FIG. 3.—Changes in spleen size expressed in percent body weight (1) in nonimmune and (2) in immune and challenged mice (series 9, see table 1 for details). Each data point is from one mouse.

tissue. There were strikingly fewer degenerative changes, less lymphocyte depletion and less stimulation of the myeloid tissue. Parasites were extremely scarce.

By the 3rd day, the lymphatic nodules were enlarged and the lymphocytopoietic centers contained a few mitoses. Erythropoiesis was practically normal in amount. By the 6th day, the white pulp and mitotic figures within the lymphatic nodules had increased still more, and reticular cells were mobilizing into large lymphocytes. Some myelopoiesis in the red pulp involved mitoses in the early red cell series. Small areas of lymphoid depletion occurred. These changes progressed to a peak on the 10th day. Beginning on the 13th day, mitotic activity decreased in both the lymphatic and the myeloid tissues. By the 20th day, all activities had regressed markedly but the enlarged transitional zones indicated the extent of the previous hyperplasia of the white pulp (pl. 2, fig. 5) and myelopoiesis was mainly localized to the periphery of the spleen near the capsule (pl. 2, fig. 5). Other evidences of stimulation are described in the following paragraph.

Immunized and challenged mice.—The spleen on the 1st day of the challenging infection showed unmistakable evidences of previous stimulation. It was somewhat larger (text fig. 3). The periarterial sheaths in cross section were smaller but were more numerous than in normal mice. Thus, proliferation of the white pulp was sufficiently intense to maintain the white pulp red pulp ratio as described in normal mice. In spite of these changes, the spleen was more or less quiescent. The nodules were normal in appearance except that the lymphocytes were not as tightly packed and there was generally a reaction center (area of reticular cells and a few lymphocytes) or a much larger than normal lymphocytopoietic center composed chiefly of large lymphocytes. In either case, small lymphocytes were numerous in the white pulp and in the transitional zone. Only a few lymphocytes were dividing. Erythropoiesis was greatly reduced. Groups of myeloid cells were localized in small foci and some of the larger stem cells were dividing. Throughout the red pulp, small focal areas consisted of a central collection of a few free macrophages and

medium polyblasts surrounded by small lymphoid cells. They were similar to those seen in the virulent infections (cf. pl. 1, fig. 4) but did not contain parasites.

Two series of mice (6 and 9) were immunized with culture forms and challenged with virulent trypanosomes subcutaneously. The reactions in the lymphatic tissue were similar in the two series, but lymphoid hyperplasia was less and myelopoiesis was more intense in series 6 than in series 9. Only an occasional leishmania showing varying degrees of degeneration was seen. In both series, the opposing processes of lymphocyte proliferation and lymphocyte depletion started on the 1st day after the challenging dose and were marked on the 3rd day. At this later time, many reticular cells were mobilizing into large lymphocytes. By the 5th day, the enlarged periarterial sheaths showed intense mitotic activity in the reticular cells and larger lymphocytes. Concomitantly, some areas of the nodules were depleted of lymphocytes. During the ensuing 5 days, the spleen became larger (text fig. 3). The lymphatic tissue decreased while the red pulp increased in amount (pl. 2, fig. 7 and 8).

By the 10th day, the white pulp which was relatively greatly reduced contained much lymphorrhexis, phagocytosis of cellular debris and some areas depleted of lymphocytes. The nodules were to a large extent lymphocytopoietic centers. In spite of this reduction of lymphatic tissue, intense reparative activity was present. It involved the mobilization of reticular cells into large lymphocytes and the active mitotic proliferation of reticular cells and of large and medium lymphocytes. The greater lymphoid hyperplasia and less lymphocyte depletion in series 9 than in series 6 can be seen by the larger nodules and the larger content of lymphocytes in the transitional zones in figure 8 than in figure 7 of plate 2. This was undoubtedly an expression of a higher degree of immunity due probably to the fact that the mice in series 9 were older.

Concomitant with these reactions in the lymphatic tissue, myeloid activity in the red pulp in series ó was intense by the 5th day, and areas of erythropoiesis containing many megakaryocytes were strikingly increased in size. By the 10th day, myeloid tissue constituted the major portion of the enlarged red pulp (pl. 2, fig. 7). Mitotic activity of proerythroblasts and of early stem cells (hemocytoblasts) was maintained at a high level. In addition, reticular cells of the red pulp were mobilizing into hemocytoblasts (or large lymphocytes). In series 9, myelopoiesis only slightly increased through the 21st day (pl. 2, fig. 8). The varied myeloid activity was probably another expression of the differences between the two series of mice.

In some areas, late in the infection, plasma cells were found in profusion in the red pulp. Many of these seemed to be developing from lymphoid cells of different sizes and at times from cells of the erythroid series (pl. 1, fig. 3).

Peripheral lymph nodes

Normal mice.—The structure of the diffuse lymphatic tissue of the cortex varied markedly. In some mice, the cortex was sharply differentiated from the medulla and nodules were present in the cortex. In other mice, the diffuse tissue was reduced in amount, the boundary between cortex and medulla was ill-defined, and no nodules were present. Mitoses in a few lymphocytes, minimal lymphorrhexis and occasional reticular cells filled with lymphocytic debris could be found in all sections. The right inguinal and lumbar-aortic

nodes were essentially similar histologically.

Virulent infection.—Lymphocyte depletion was reflected both by a reduction in the relative thickness of the cortical layer and by an irregular reduction in the density of the lymphocyte population of the cortex and the medulla. Both were evident on the 2nd day. On the 3rd day, depletion was accompanied by an almost generalized mobilization of reticular cells. Some were dividing and others were transforming into large lymphocytes. Large lymphocytes were also dividing. In addition, the 3rd day marked the appearance of parasites in the reticular cells and in the fat cells of the connective tissue surrounding the node. On the 4th day, cell destruction and phagocytosis were seen in small areas containing a few free macrophages, heterophils, polyblasts and lymphocytes. Some polyblasts contained cellular debris but no recognizable parasites. Lymphocytes were degenerating. These processes are probably a part of the picture of the acute adenitis of Chagas' disease, as described by Mazza et al (1942), and are similar to the inflammatory-like foci previously described in the red pulp of the spleen.

The processes initiated on the 3rd day developed as follows: Lymphocyte depletion progressed more or less continuously to a marked peak during the last few days. Concomitantly, cellular (largely lymphocytic) debris was intensely phagocytosed. Phagocytosis reached its height at about the 7th day and rapidly regressed thereafter, apparently because the bulk of the cellular debris had been removed and digested. The mobilization of reticular cells varied from time to time but was still evident during the terminal stages of the infection. Mitotic activity was intense at first but decreased during the last two days. It largely involved reticular cells at the beginning but was chiefly restricted to large and medium lymphocytes later. Initially, macrophages were derived almost entirely from reticular cells but, from the 6th day on, they were also derived heteroplastically from agranulocytes. A few of the earlier stages in this cytogenesis, i.e., monocytoid lymphocytes intermediate between lymphocytes and monocytes, are depicted in plate 3, figure 2. From the 8th day on, lymphocytes of all sizes transformed into plasma cells. Many of the macrophages, both with and without parasites or phagocytosed debris, were cyst-like in appearance due to a condensation of cytoplasm around a large central vacuole. They were similar to the cells described by Torres and de Azevedo (1929a and b) except that most of them only contained one nucleus (pl. 3, fig. 1). A probable early developmental stage is illustrated in plate 3, figure 2 (cells marked Mac).

Late in infections, erythropoietic foci occurred occasionally (pl. 3, fig. 1) and were almost always associated with plasma cells.

Parasites increased slowly in number from the 4th day to the end of the infection. They were ordinarily found in reticular and littoral cells, but were also found during the latter part of the infection in inflammatory macrophages and polyblasts. As in the spleen, only a few of the parasites appeared damaged.

Immunized and challenged mice.—Reactions in the nodes of these mice were largely proliferative and reparative with no marked cell destruction. Thus, on the 6th and 7th days, there was little lymphocyte destruction or depletion, but there were many lymphocytes and a few reticular cells dividing and a marked transformation of reticular cells into large lymphocytes. Some of the medullary sinuses were filled with free swollen macrophages which only occasionally

contained phagocytic debris. Some of the macrophages arose heteroplastically as evidenced by a continuous series from them through medium polyblasts to agranulocytes. The medulla also contained numerous plasma cells. At no time were parasites found.

Bone and bone marrow

Normal mice.—All sections were taken from the proximal half of the femur. There was always an active process of endochondral ossification.

Virulent infection.—Parasites were found in various stromal cells in the invading connective tissue of the zone of endochondral ossification and in endostial and periostial cells as early as the 3rd day. They increased in number with time and were numerous after the 10th day. They were found predominantly in reticular cells of the marrow, rarely in cartilage cells and more rarely in bone cells (pl. 4, fig. 2 and 4). Infection of bone and cartilage cells was limited to areas adjacent to the connective tissue invading the zone of endochondral ossification and was never deep in the cartilaginous or calcified tissue. Infection of bone cells presumably occurred before the cells were surrounded by bone matrix.

The first noticeable reaction of the bone marrow to the infection occurred on the 4th day when stem cells and mitotic figures increased. Superimposed on this activity by the 7th day was an extensive cytolysis among the smaller lymphoid cells and active phagocytosis of the resulting cellular debris. In addition, localized areas of inflammatory cells included many parasites in large macrophages and intermediate polyblasts. From the 9th day on, stem cells increased in number along with epithelial-like areas of hemocytoblasts. Large numbers of mitoses occurred in the reticular cells, hemocytoblasts and smaller free cells. Hemocytoblasts began to occur in islands developing from the endostium and filled most of the bone marrow cavity by the 14th day. Erythropoiesis and granulopoiesis varied in amount, but both were markedly stimulated.

Immunized and challenged mice.—Our material was very limited, but suggested a mild stimulation of both series (granulopoiesis and erythropoiesis) similar but milder than that found during the initial infection. No parasites were found.

Thymus

Normal mice.—The structure of the thymus of the different groups of mice was comparatively uniform and showed no evidences of changes that could be associated with age.

Virulent infection.—Few parasites or histological changes were observed in animals before the 6th day of the infection. At this time, the thymus of most animals showed varying degrees of lymphocyte (thymocyte) depletion, especially of the cortex, degenerating lymphocytes in the medullary zone, and the mobilization of reticular cells into macrophages which contained both cellular debris and leishmaniform parasites. In the particular tissue shown in plate 4, figure 1, there was a fairly sharp differentiation between reticular cells containing debris and those containing parasites. Lymphocyte depletion was sometimes pronounced at 7 days and was usually advanced by the 9th day. During this stage of the infection, lymphocytes practically disappeared from extensive areas of the reticular syncytium, especially in the cortex (pl. 4, fig. 3).

In general, parasites invaded reticular cells at the outer periphery of the cortex first, throughout the cortex next, and the medulla last. This sequence may explain why lymphocyte depletion was

first seen in the cortex and why there were fewer lymphocytes in it at certain stages than in the medulla. Only during late stages did lymphocytes divide and develop into plasma cells. Macrophages mobilized from the reticular cells at first but developed from lymphocytes during the last few days. At no time was there a marked transformation of reticular cells into large lymphocytes. The few cases seen involved reticular cells near blood vessels. This finding is possibly connected with the fact that reticular cells near the blood vessels are more likely to be of mesenchymal origin than reticular cells in the parenchyma.

Immunized and challenged mice.—In general, the thymus of superinfected mice showed the same type of changes found in initial fatal infections except that damage was minimal. The first evidences were seen on the 6th day and involved an increased number of dividing lymphocytes and the mobilization of an occasional reticular cell. This picture was associated with lymphocyte depletion in a few areas and with the development of a number of plasma cells on the 7th day. By the 8th day, when parasites were first found in a few reticular cells, there was considerable lymphocyte depletion, many mitoses, the development of plasma cells, and some heterophil infiltration of the medulla. The lymphocyte depletion was minor as compared to that in mice initially infected with virulent parasites. There was little cellular debris or phagocytosis by the reticular cells. Mild inflammation was present in the fatty connective tissue surrounding the organ, but no parasites were found in it.

Adrenals

Virulent infection.—Parasites were not found in appreciable numbers in the adrenals until the 6th or 7th day following injection of a large number of virulent parasites. They occurred chiefly in littoral cells but were not associated with any inflammatory changes. By the 9th day, small inflammatory foci occurred close to the medulla in the zona reticularis in what were probably littoral cells and glandular cells. It is interesting that the foci were not necessarily associated with the presence of identifiable parasites and that they occurred later in the adrenals than in the liver.

Immunized and challenged mice.—After the challenging infection, the adrenals were essentially normal. Neither parasites nor inflammatory foci were found.

Liver

Normal mice.—The liver contained very few lymphoid accumulations in the periportal connective tissue. When they occurred, they were not extensive and did not increase as a result of infection.

Virulent infection.—Parasites and lesions were first found in the liver on the 3rd or 4th day after heavy inoculations. The lesions consisted of small foci of inflammatory exudate cells, including a few medium polyblasts, heterophils, lymphoid cells and free macrophages. (A focus from a later stage is shown in pl. 5, fig. 2.) These foci were irregularly placed and increased gradually in number as the infection progressed.

A striking phenomenon was the differential content of parasites and the fate of the parasites in the Kupffer cell and the inflammatory macrophage—although neither type of cell appeared to be aggressively phagocytic in the nonimmune host. Thus, on the 3rd day, Kupffer cells occasionally contained one to several leishmania. Progressively thereafter, more of them became infected and contained more and more parasites. On the 6th or 7th day, and especially during the ensuing rapid rise in parasitemia, many were enormously

swollen with apparently normally developing parasites (pl. 5, fig. 1). The parasites did not seem to be digested and were evidently nonirritating as long as the host cell remained intact. In contrast, markedly fewer parasites were found in the imflammatory macrophages in the small inflammatory foci late in the infection and frequently appeared to stain abnormally and to be obviously damaged (see especially those in the upper left of pl. 5, fig. 2). Such inflammatory foci were evidently defense centers against the infection, but were inadequate to cope with the infection in the nonimmune host (see also Pizzi, Rubio and Knierim, 1953 and 1954).

Mazza and Jörg (1953a) in their study of puppies dying of Chagas' disease suggested that the nodular foci arise by the following sequence: (1) A hematogenous phase following the initial local tissue focus of infection. (2) An acute hepatitis with intense leucocytic infiltration followed by an accentuation of the granulomatous process. (3) Chronic hepatitis in which the post-inflammatory histiocytosis is condensed from a diffuse process to a nodular one with many leishmania and intense round cell infiltrations. (4) A hematogenous phase due to the discharge of the leishmania from the nodules into the blood stream. (5) A chronic hepatitis without leishmania characterized by multiple foci of round cells with some hypertrophied or degenerating histiocytes.

The local inflammatory lesions which we found were similar to their 3rd stage except that they were less circumscribed and contained more heterophils. As pointed out above, however, the process preceded the rise in parasitemia and was not associated with a preceding diffuse inflammatory process, either acute or chronic, in the liver. Also, in our material the accentuation of the parasitemia as postulated in their fourth stage led to a heavy Kupffer cell infection without much increase in the focal lesions.

Immunized and challenged mice.—The liver was essentially normal during the first 4 days after the challenging infection. In other words, there were no residual focal lesions. A few small accumulations of lymphoid cells were found on the 5th day, and a few foci of inflammatory cells were scattered throughout the tissue on the 7th day. One to several parasites were occasionally found in mononuclear exudate cells and in nearby Kupffer cells. By the 8th day, the inflammatory foci were larger and some contained damaged cells and many heterophils. At this time, parasites (some looked damaged) were found in the mononuclear exudate cells and only rarely in Kupffer cells.

As compared to the initial infection, both foci and parasites were less numerous, but relatively more parasites occurred in inflammatory macrophages in the foci than in the Kupffer cells. The parasites appeared abnormal and were probably being digested. Antibodies acquired during immunization, as reported by Pizzi et al (1954), undoubtedly aided in the phagocytosis of the challenging dose ot virulent parasites.

Skeletal muscle

Virulent infection.—Parasites were first found in muscle fibers of the subcutaneously injected thigh on the 3rd day and did not markedly increase in number thereafter. On the 6th day, parasites began to appear in the interstitial connective tissue and increased in this tissue through the 9th day. Thus, leishmaniform parasites occurred first in muscle cells, but later exhibited a marked preference for interstitial connective tissue cells. Parasites also occurred in medium polyblasts and macro-

phages when inflammation was present. A few inflammatory cells were found on the 7th day and afterwards, but inflammatory reactions were mild except occasionally when a marked diffuse infiltration of inflammatory cells was associated with degeneration and necrosis of muscle fibers.

Immunizing infection.—No inflammation or parasites were found in mice inoculated with low virulent culture forms.

Immunized and challenged mice.—Local inflammatory foci occurred in the fat tissue surrounding the muscle before they were found in the muscle in this group of mice. They roughly paralleled those in the fatal initial infections both in regard to the time of their onset and their mild character. Very few parasites were seen at any time.

Heart

Virulent infection.—Parasites in small numbers were found in the muscle fibers from the 5th to the 7th day. They increased in number until they were frequently numerous by the 9th day. Inflammation, beginning on the 9th day, varied from a mild diffuse cellular exudate to local areas of edema and many inflammatory cells. In one animal, inflammation was especially severe in the loose connective tissue near the atrioventricular valves. The inflammatory process seemed to be correlated with long infections rather than with the number of parasites present. It was generally present only in animals which survived longer than 9 days.

Immunizing infection.—In mice inoculated with low virulent culture forms, the heart sections were practically normal. There were only a very few parasites and a very few small inflammatory foci around the 15th day in some animals.

Immunized and challenged mice.—Parasitization and histological changes in the immune heart after the challenging dose closely paralleled those seen in the pathogenic initial infections. The processes, however, were milder. A few parasites in the muscle fibers of the heart and several small aggregations of small mononuclear cells were first seen on the 7th day. Parasites were not the center of any noticeable inflammatory process. For the following 3 days, the inflammatory foci continued to be mild and parasites scarce. From 2 weeks to a month and a half after the challenging dose, some mice died with a mild degree of myocardial inflammation but with only a few parasites present. The number that died, however, was variable. Among 20 mice, 1 to 3 might die in one series and 9 or 10 in another.

Lungs

Virulent infection.—Tissue invasion of the lungs was apparently a late manifestation of the infection. Leishmania were first found on the 7th day in fairly large numbers within the septal cells (local macrophages). They developed and increased greatly in number during the ensuing two days but were not associated with any exudation of inflammatory cells in the alveoli.

Immunized and challenged mice.—At no time were many parasites found in the lung, but mild inflammation, as indicated by the presence of inflammatory exudate cells in the alveoli, was found after the first week.

Intestine

Virulent infection.—Some parasites were found from the 3rd day on in fixed macrophages of the intestinal wall (pl. 5, fig. 3). Most of them seemed to develop normally. From the 6th day on, developing parasites were also seen in the smooth muscle fibers of the intestine. No important inflammatory reac-

tions or other lesions were observed.

Immunized and challenged mice.—No parasites or lesions were found.

DISCUSSION

The designation of the present strain of *T. cruzi* as reticulotropic by Badínez (1945) indicates one of the chief cell preferences of the parasite and is probably as appropriate a name as possible. It is not, however, completely descriptive because other connective tissue cells also become infected. Among infected macrophages not derived from reticular cells were adventitial cells of the loose connective tissue and inflammatory macrophages and histiocytes of the skin and among those which are possibly not reticular cells were the littoral cells of the liver and adrenals. Among nonphagocytic connective tissue cells, the only ones comparable to macrophages as sites of infection were fat cells of the loose connective tissue. Fibroblasts were infected less frequently. In addition, cell preference often changed as the infection proceeded. Thus, early initial infection of the skin involved fibroblasts and histiocytes, whereas later initial infection involved almost exclusively inflammatory macrophages of both local and hematogenous origin. Small intermediate polyblasts frequently contained parasites. In heart and skeletal muscle, muscle cells were generally the first to be infected but, later, macrophages of the interstitial connective tissue were the chosen sites. Lymphocytes, hemocytoblasts and lymphoid cells of the myeloid series were not infected. It is remarkable that parasites were not found in true endothelial cells lining the nonsinusoidal vessels. They were, however, found in the endothelial cells of the smaller splenic veins. If the latter cells actually phagocytose the parasites, they are similar to the cells in the spleen of Indian monkeys which phagocytose malarial parasites, according to Taliaferro and Mulligan (1937). Parasites were not found in heterophils except when the host was injected subcutaneously with low virulent culture forms. Somewhat similarly, Taliaferro and Bloom (1945) found that heterophils in areas of subcutaneous injections contained erythrocytes infected with plasmodia.

The chief connective tissue reactions occurred in the loose connective tissue of the skin (in animals infected subcutaneously) and in the reticular organs. Experimental Chagas' disease in C_3H mice resulted in a severe drain on the lymphoid and myeloid systems including lymphocytes and, to a less extent, the free stem cells of the myeloid series. The same type of depletion occurred in animals immunized with low virulent culture forms but was adequately met by the proliferative and reparative reactions of lymphatic and myeloid tissue.

Greatest attention will be given to the lymphoid system as it was most affected. Lymphocyte depletion was due, in part, to the lymphocytocidal effects of the infection and, in part, to the transformation of lymphoid cells into macrophages. The proliferative and reparative reactions were fundamentally identical in the ineffective response of nonimmune mice and the effective response of immunes. They included in part (1) the often intense and widespread mobilization of reticular cells and their transformation into very large basophil lymphocytes,* (2) the mitotic proliferation, at first, of reticular cells and the larger lymphocytes and, later, almost exclusively of large and medium lympho-

* This is the cell which has been variously termed a large lymphocyte, an acute splenic tumor cell, a myeloblast, a lymphoblastic plasma cell, a developing or immature plasma cell, a macrohistocyte and a basophil macrophage (see Taliaferro, 1949).

cytes; (3) the mobilization of reticular cells as macrophages and later the widespread heteroplastic development of lymphocytes into macrophages; and (4) the development of all sizes of lymphocytes into plasma cells which was frequently an almost terminal phenomenon in the nonimmune animals and seldom occurred before a week in the immune animals.

The initial stimulation of lymphocytopoiesis by virulent parasites, which is followed by lymphocyte depletion in nonimmune mice but which leads to lymphoid hyperplasia in immune mice, follows closely the picture of the interplay between lymphocytopoiesis and lymphocyte depletion described by Conway (1939) in infections with *Listeria monocytogenes* and is characteristic of most, if not all, severe generalized infections. These opposed processes can be seen in the spleen and particularly in the transitional zone between the perifollicular spaces of the white pulp and the red pulp proper. In normal mice, the free lymphoid cells of the transitional zone were mostly medium lymphocytes. The first evidences of lymphoid activation of the white pulp was the loss of small lymphocytes, the mobilization of fixed reticular cells into large lymphocytes and the mitotic proliferation of reticular cells and the larger lymphocytes. The loss of small lymphocytes was due in part to their migration into the transitional zone and red pulp and eventually to their destruction or transformation into other lymphoid cells. As lymphocytopoiesis continued, the transitional zone became much broader and was filled chiefly with medium and large lymphocytes. When depletion occurred, the previously enlarged transitional zone appeared as reticulum with very few lymphoid cells in it. When reparative processes stopped depletion, small lymphocytes and eventually medium lymphocytes appeared in the transitional zone. Similar changes have been found by the senior author (unpublished work) in the spleen of chickens infected with *P. gallinaceum* and were most apparent around the Schweigger-Seidel sheaths.

In immune mice, the proliferative and reparative reactions of the lymphatic and myeloid tissues led, certainly in the spleen, lymph nodes and bone marrow, to a hyperplasia of macrophages and various lymphoid cells. The increase in these cells was shown by the fact that the spleen and lymph nodes increased in size and continued to contain the same or an even greater numbers of cells per unit area while the bone marrow contained far more lymphoid cells per unit area. New lymphocytes and myeloid stem cells arose chiefly by mitotic proliferation and also, in the case of the larger cells, by the direct transformation of reticular cells.

Many free macrophages, and, to a less extent, fixed reticular cells were destroyed by the parasites and the infectious process. Replacement of the cellular reticulum and the development of new reticulum was associated with the mitotic proliferation of reticular cells. Whether any reticular cells developed from free cells could not be decided from our material. Free macrophages arose by mitotic division of unengorged reticular cells and by the heteroplastic development of agranulocytes. In this connection, it is not unusual for authors to describe as reticuloendothelial hyperplasia situations in which the reticular cells of the stroma of lymphatic organs become clearly visible because of the loss of lymphocytes from the stroma. Such tissues, however, have actually lost lymphocytes and have not gained reticular cells per unit area. (See discussion in Conway, 1939.) We believe that a part of the histiocytic hy-

perplasias described by Mazza et al (1942) and Mazza and Jörg (1943) in Chagas' disease belong in this category.

The functional significance of lymphoid hyperplasia probably lies in the fact that it provides a mesenchymal reserve from which (1) lymphocytes and various other cells are produced, (2) macrophages are developed for the removal of cellular debris resulting from the destruction of lymphoid cells and of various disintegrating parasitized cells and parasites, and (3) macrophages are supplied in immune animals which are functional in acquired immunity. In regard to the last mentioned function, Pizzi et al (1953 and 1954) have recently found that acquired immunity in rats and mice to the same strain of *T. cruzi* used in the present work was associated with phagocytosis chiefly by inflammatory macrophages in the focal and diffuse inflammatory reactions throughout the tissues—probably in cooperation with antibodies. In addition to the functional aspects of the production of new macrophages, their production may also be in part a somewhat stereotyped and fundamentally adaptive process which, in the nonimmune host, furnishes the cells of choice for infection and reproduction of the parasite.

One of the central problems in immunity to *T. cruzi* is the mechanism whereby the macrophage changes from a site in which the parasite grows in the nonimmune host to a site in which the parasite is destroyed in the immune host. Our observations and those of Pizzi et al (1953 and 1954) indicate that this change is largely due to the acquisition of antibodies, but that, in addition, the inflammatory macrophage is more effective than the fixed macrophage in the disposal of parasites.

The situation in infections with *T. cruzi* is similar to that in certain malarias such as *Plasmodium brasilianum*. In the nonimmune host, the basic reproduction of the malarial parasite is constant (except temporarily during the crisis) and a constant percentage of the parasites are destroyed (innate immunity). After the host acquires immunity, phagocytosis and the destruction of parasites are so intense that the infection is reduced to a low grade. The continued survival of a few parasites in the immune host is probably associated with a small proportion of phenotypically but not genotypically resistant organisms (cf. Taliaferro, 1948).

Less is known about the functional significance of the intense stimulation of erythropoiesis. As far as we could find, no marked change in the number of red cells occurred as a result of infection in either immune or nonimmune animals, and reticulocytes only reached 10% at their peak during terminal stages of fatal infections. In some of the spleens during late stages of the infection, erythropoiesis appeared to stop at about the polychromatophilic erythroblast stage and, during even later stages of the infection, the basophil members of the erythroid series often developed into plasma cells. If the recent contention of a number of investigators that antibodies are formed by the plasma cell series is substantiated (review by Taliaferro, 1949), erythroid hyperplasia by being redirected to plasma cell formation may become of great functional significance. The erythroid cells, however, are not the only source of plasma cells. Many arise from lymphocytes of various sizes.

Numerous authors, particularly Mazza (see Mazza and Jörg, 1935b), have emphasized the inflammatory aspects of Chagas' disease. The relation of parasites to the inflammatory reactions in the various tissues studied in this work is complex. A sharp distinction should be made between the inflammatoid re-

actions in the lymphatic and myeloid tissues (spleen, lymph nodes, thymus and bone marrow) and the inflammatory reactions in other sites such as the skin. The first mentioned reactions were directly associated with the destruction of lymphocytes and other lymphoid cells and were correlated with the degree of tissue invasion. Hence, they were intense during late stages of virulent initial infections but were always minor in superinfected animals. The inflammatory reactions of the organs, other than the lymphatic and myeloid organs, on the other hand, exhibited about the same degree of activity for the first week or 10 days and became more marked in immune mice in spite of low grade infections. Thus, Pizzi (1951) found various degrees of local or diffuse myocarditis in a small percentage of mice that died at irregular intervals during the long continued low grade infections following suppression with primaquine. This type of inflammation could obviously not be directly correlated with local tissue invasion by the parasite because the immune mice always had much lower grade infections than the nonimmunes. Indeed, when inflammatory reactions were most marked in the immune mice, parasites were extremely scarce.

Most inflammation in the nonimmune animals is probably nonallergic and in general follows host cell destruction and possibly the liberation of endotoxins by the parasite, whereas most inflammation in the immune animals is probably allergic and is probably associated with the production of anti-cruzi antibodies or a tuberculin-type of cellular hypersensitivity. The inflammation in the immune animal would thus be low grade at first, although sufficient to allow an inflammatory response equal to that of the nonimmune, but would later be far more intense than any encountered in the nonimmune. This explanation would encompass two seeming exceptions. In the first case, when animals with an initial infection survived over 9 days, they often exhibited more inflammation than animals which died at 9 days. The longer survival may allow hypersensitivity to develop. In the second case, when exceedingly large numbers of parasites were injected subcutaneously, more inflammation occurred at the site of the inoculation—but not at other sites—in immune than in nonimmune mice during the first week or 10 days. The immune animal may have responded more intensely in the subcutaneous tissue because of the large amount of antigen introduced but disposed of parasites so effectively that other sites did not receive much antigen.

These findings are in line with the earlier suggestion of Torres (1929) and Mazza and Jörg (1935b) and the experimental work of Muniz and de Azevedo (1947 and 1949). The last mentioned authors sensitized two rhesus monkeys with five injections of killed *T. cruzi* or lysates which they believed had been washed free of nontrypanosome proteins of the culture. When the animals were given a "shocking" injection on 8 and 33 days, respectively, after the sensitizing injections, they developed inflammatory reactions and granulomata in the pleura and a myocarditis which resembled those found in Chagas' disease. Two other control monkeys injected just once with the same material developed no lesions in the pleura or heart. The authors also succeeded in passively transferring the hypersensitivity.

The reactions in the lymphatic and myeloid tissues of C_3H mice initially infected with *T. cruzi* were similar to those described by Singer (1954) in CF1 mice infected with *Plasmodium berghei*, but neither the depletion of lymphoid cells

nor the proliferations and reparative processes were as intense in our mice. In fatal berghei infections, Singer believes that the lymphocytes, as in other malarias, form an important reserve from which macrophages are formed. As the spleen becomes almost entirely devoted to erythropoiesis, the lymph nodes, in compensation, increase their lymphocytopoietic potential with a concomitant increase in the heteroplastic development of macrophages from agranulocytes. In cruzi infections in nonimmune mice, the production of macrophages, both homoplastically by the proliferation of reticular cells and heteroplastically from agranulocytes, occurred in the spleen up to the time of the death of the host. Neither the phagocytopoietic activity nor the loss of red blood cells was as great in the cruzi infections as in the berghei infections. In cruzi infections, the spleen became involved to a very large extent in erythropoiesis in spite of the absence of any pronounced anemia. These findings suggest that the erythropoietic activity in the spleen may largely represent the genetically conditioned response of the mouse to severe infection in general.

CONCLUSIONS

A study of the connective tissue reactions has been made of a highly inbred C_3H strain of mice infected with a standardized virulent reticulotropic (Tulahuen) strain of *Trypanosoma cruzi*. Tissues were studied from nonimmune mice infected with the virulent strain, from mice immunized with low virulent culture forms and from immunized mice challenged with the virulent strain.

1. In general, reproducing leishmaniform parasites were found (a) often in macrophages including reticular cells of the reticular organs, littoral cells of the liver and adrenals, adventitial cells of the loose connective tissue, histiocytes of the skin and inflammatory macrophages in various tissues, (b) about as frequently in fat cells of loose connective tissue, (c) less often in fibroblasts during early infection, (d) much less often in muscle cells, (e) rarely in cartilage and (f) extremely rarely in bone cells.

2. Although most virulent parasites apparently developed normally in free and fixed macrophages (= reticular cells, littoral cells and histiocytes), a few were digested, especially in inflammatory macrophages, in the nonimmune mouse. Their phagocytosis and destruction were markedly enhanced in the immune mouse due to the development of acquired immunity. Low virulent culture forms were avidly destroyed, especially by the inflammatory macrophages, in both nonimmune and immune mice.

3. The most pronounced connective tissue reactions occurred in the reticular organs and consisted of opposing processes leading to (a) the depletion of lymphoid cells and (b) the cytogenesis of lymphoid cells from the cellular reticulum and by mitotic proliferation. Less pronounced was the loss of macrophages and development of new macrophages homoplastically by the mitotic proliferation of reticular cells and heteroplastically from agranulocytes. In nonimmune animals, the destructive processes eventually predominated whereas in immune animals the reparative and proliferative processes led to marked lymphoid, myeloid and macrophage hyperplasias. The immune animal was thus supplied with extra mesenchymal reserves for the production of macrophages and probably antibodies.

4. Focal and diffuse inflammatory processes occurred throughout the tissues. In nonimmune animals these increased in number as the fatal infection

progressed whereas in immune animals they were apparently allergic in character and their intensity was not correlated with the degree of infection. Allergic inflammatory processes in the myocardium possibly accounted for the death of some immune animals which had successfully suppressed the challenging infection to a very low grade.

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COMPARATIVE STUDY OF HOST RESISTANCE OF GUINEA PIGS AND RATS

I. VIRULENCE OF *DIPLOCOCCUS PNEUMONIAE* AND *BACILLUS ANTHRACIS* FOR GUINEA PIGS AND RATS

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A comparative study of possible mechanisms of resistance of two animals, the guinea pig and the white rat, to two pathogens, *Bacillus anthracis* and *Diplococcus pneumoniae*, has been undertaken with the hope of contributing to the knowledge of species, or ultimately organ resistance, or, conversely, susceptibility to a given microorganism. The two hosts vary markedly in their resistance to the selected pathogens. Ultimately from findings of work of this nature it may be possible to measure the resistance of man to a given parasite and conversely, the virulence of a given parasite for man by logical in vitro procedures. At present only empirical techniques such as the coagulase test with staphylococci are available for measuring in vitro the virulence of a parasite for man.

The first studies of this general project will be reported in this paper. The virulence of the selected strains of the two parasites, *D. pneumoniae* type I and *B. anthracis*, has been evaluated by a determination of the lethal effect of the pathogens for the two host species and the rate of clearance of the organisms from the blood stream and organs of the two hosts. The results of attempts to lower host resistance by splenectomy, or by so-called reticulo-endothelial blocking procedures will also be reported.

Comparative studies of host resistance obtained by our associates to be published include investigations of the effect of serum,[1] extracts of tissues, particularly liver[2] and leukocytes,[3] on the two parasites and the effects of the pathogens or fractions of such on the metabolism of leukocytes,[4] or on the activities of adenosine triphosphatase from leukocytes of the two hosts,[5] the rat and the guinea pig. These reslts will be submitted in subsequent papers.

GENERAL METHODS AND MATERIALS

Guinea pigs used in these studies were obtained from local dealers and were not definable in terms of a genetic strain. On the average these animals weighed about 300 g. White rats were of the Sprague-Dawley strain and weighed about 150 to 200 g.

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The strain of *D. pneumoniae* type I used was obtained from Dr. C. P. Miller of the University of Chicago, Department of Medicine, and was designated by him as strain A-5. In this laboratory it has been carried as strain 76. It was routinely maintained by passage through rats at 3-week intervals. The organism was recovered by plating out the heart blood, then culturing the recovered organism in brain heart infusion broth for subsequent storage at 5 C. Subcultures were made as required and incubated at 37 C for 6 hours. Experience indicated that with the techniques employed, this culture was in the logarithmic phase at 6 to 8 hours, and contained from 10^8 to 10^9 organisms per ml. The amount necessary to kill mice was usually 10^{-7} ml of such a culture diluted in gelatin (0.5%)-saline (0.85% NaCl) and containing from 20 to 50 organisms.

A culture of *B. anthracis* (Vollum strain) was obtained from Dr. Francis B. Gordon of Camp Detrick. Spores of this organism kept at 5 C were inoculated into human serum broth and incubated for 24 hours at 37 C prior to use.

RESULTS

Lethal action of pathogens.—Logarithmic dilutions of the cultures of the two pathogens, *D. pneumoniae* or *B. anthracis*, were inoculated intraperitoneally into the two hosts, guinea pigs or rats. The results of repeated tests over several years and done by different individuals were quite consistent. Table 1 gives the results of a typical experiment and indicates that the guinea pig is about a million times as resistant to intraperitoneal injections of *D. pneumoniae* type I strain 76 as is the white rat. In a similar manner the results of a typical experiment with *B. anthracis* indicate that it requires about 1000 times as many organisms of the strain used to kill a white rat as a guinea pig. The difference in susceptibility of guinea pigs and rats to *D. pneumoniae* is less generally known than the widely recognized difference in susceptibility of rats and guinea pigs to *B. anthracis*, although comparative studies have been made as early as 1912.[6]

TABLE 1.—*Pathogenicity of* D. pneumoniae *type I and* B. anthracis (*Vollum strain*) *for rats and guinea pigs injected intraperitoneally.*

	Number of pneumococci	Dead/Inoculated	Number of *B. anthracis*	Dead/Inoculated
Rats	20	3/5	33,000	0/5
	200	4/5	330,000	0/5
	2,000	5/5	3,300,000	2/5
	20,000	5/5	33,000,000	5/5
Guinea pigs	200,000	0/5	3	0/5
	2,000,000	2/5	33	0/5
	20,000,000	2/5	330	1/5
	200,000,000	4/5	3,300	2/5
			33,000	5/5

The difference in susceptibility of rats and guinea pigs to *D. pneumoniae* injected by various routes was determined. The order of difference in susceptibility of these two hosts was the same when measured by the lethal effects of intracardial and intraperitoneal inoculations. By intradermal injection the approximate LD_{50} dose for guinea pigs was of the order of 10^{-1} ml of the test culture and 10^{-3} ml for the rat. When the bacteria were injected intracranially, the lethal dose for guinea pigs and rats was 10^{-3} and 10^{-6} ml, respectively. In these tests five animals were used for each dose and each site of inoculation.

Blood and tissue clearance of D. pneumoniae.—Having determined that the rat was nearly a million times more susceptible to *D. pneumoniae* type I strain 76 than the guinea pig and at least 1000 times more resistant to the test strain of *B. anthracis*, a program was begun to search for the underlying mechanisms responsible for these marked differences in host resistance.

To determine the rate of clearance of inoculated organisms from the blood and tissues of animals, 20 guinea pigs and rats were injected intracardially with about 3.8×10^7 pneumococci. At each time interval indicated in table 2,

6. Truche, C. and Cotoni, L. 1912, Etudes sue le pneumocoque. V. Virulence des pneumocoques, humains et animaux, pour le lapin et le cobaye, Ann. Inst. Past. 26: 530–535.

TABLE 2.—*Rate of clearance of pneumococci from blood and tissues of rats and guinea pigs.*

Liver		Spleen		Kidney		Brain		Blood	
Rat	G.P.	Rat	G.P.	Rat	G.P.	Rat	G.P.	Rat	G.P.
15 min.:									
486,000*	21,200	10.2 M	250,000	151,000	13,000	76,000	4,000	486,000	27,000
300,000	92,000	3.2 M	550,000	81,000	84,000	29,000	10,000	350,000	342,000
30 min.:									
164,000	8,000	2.0 M	16,000	29,000	11,000	3,000	1,000	196,000	12,000
136,000	9,000	2.6 M	2.7 M	88,000	12,000	10,000	X	162,000	14,000
60 min.:									
121,000	1,000	600,000	6,000	95,000	500	5,000	7,000	4,000	6,000
113,000	4,000	640,000	21,000	25,000	2,000	3,000	1,000	50,000	30,000
2 hr.:									
12,000	10	1.3 M	9,000	X	1,000	1,000	100	47,000	1,000
51,000	10	440,000	2,000	33,000	1,000	1,000	100	76,000	9,000
8 hr.:									
440,000	84,000	90,000	97,000	50,000	264,000	1,000	216,000	147,000	3.2 M
9 M	69,000	6.3 M	35,000	741,000	270,000	70,000	77,000	166,000	90,000
13 hr.:									
16.7 M	3,780	15.8 M	11,000	438,000	3,240	2.8 M	1,200	2,700 M	390
4.0 M	220	10.1 M	460	17 M	1,460	140,000	370	20 M	24,000
24 hr.:									
1.6 M	18,000	24.6 M	37,000	16.6 M	4,230	4.9 M	42,000	13.1 M	3,100
1.2 M	17,000	17.9 M	5,400	23 M	X	1.6 M	8,000	4.4 M	370,000
30 hr.:									
378,000	3,200	7.4 M	4,800	5 M	5,400	1.5 M	1,440	26 M	1,620
D	1,110	D	6,400	D	70,000	D	5,400	D	200
51 hr.:									
D	1,200	D	5,000	D	960	D	270	D	X
D	16,000	D	480,000	D	702,000	D	3,800	D	1,360
100 hr.:									
D	20,000	D	82,000	D	103,000	D	2,150	D	3,780
D	10	D	130	D	12,000	D	550	D	0

M, millions.
X, contaminated.
D, dead.
* Corrected values—number of organisms per gram of specific tissues; 38 million organisms inoculated into each animal.

two animals were sacrificed and the number of viable organisms present in the blood and certain of the tissues determined. It will be noted from the results of a typical experiment given in table 2 that in the rat there was a general increase in number of organisms in both the blood and organs. Rat spleens, 15 minutes after inoculation, contained 1000 times or more organisms than were found in the blood. The number of organisms increased in the blood and tissues until the rats died, usually within 24 to 36 hours.

In general, guinea pigs tended to keep down the number of organisms in both the blood and tissues. The number of pneumococci in the spleen, liver, and blood of guinea pigs at 100 hours was usually relatively small, and in 4 to 6 days the blood and tissues were sterile.

Clearance from lung.—Guinea pigs and rats were exposed to an aerosol of *D. pneumoniae*, using a simple technique previously described.[7] Guinea pigs and rats both received a pulmonary inoculum of 4 to 5 thousand organisms, as shown by plate counts on lungs of animals sacrificed immediately after exposure to the aerosol. In the case of the resistant guinea pig, the viable pneumococci in the lungs rapidly decreased in number, occasionally reaching a zero level within 24 hours. Although the organism in the rat lung tended to decrease during the first 6 hours, there was

7. Ames, A. M. and Nungester, W. J. 1949, The initial distribution of air-borne bacteria in the host. J. Infect. Dis. **84**: 56–63.

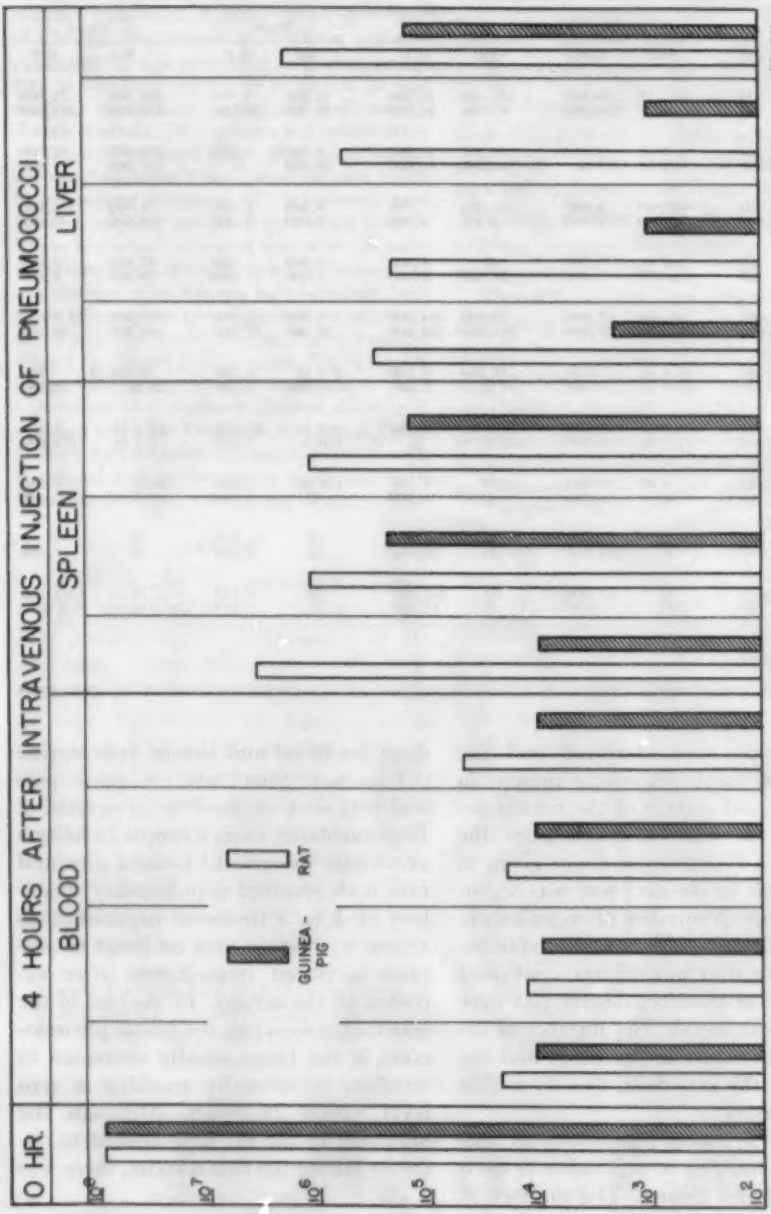

FIG. 1.—Pneumococci in blood and tissues of rats and guinea pigs inoculated intracardially with 10^8 pneumococci type I.

then a marked increase in pneumococci. However, after several days the lungs were again free of organisms.

Clearance of blood after splenectomy or "blocking" of reticulo-endothelial cells with 10% saccharated iron oxide.—From the results described it is apparent that the fixed phagocytic cells of the reticulo-endothelial system of both the guinea pigs and rat are active in removing this test organism from the blood. For this reason it was decided to determine whether or not splenectomy or blocking of the reticulo-endothelial system by injection of colloidal particles would affect the clearance of the blood and tissues of organisms in the two hosts.

The spleens of guinea pigs and rats were removed and, after 4 days, these animals were injected intracardially with 1 ml of a 5- to 6-hour old culture of the virulent *D. pneumoniae.* Four hours later the animals were sacrificed and bacterial counts were made on blood, spleen, and liver tissue. Also, two groups of 20 animals each were injected with 5 ml intraperitoneally, or 1 ml intracardially, of a 10% saccharated iron oxide suspension. Sixteen hours later the animals were injected intracardially with 1 ml of a 6-hour pneumococcus culture.

The results of these experiments are presented in a bar graph (fig. 1). From 19 to 23 animals were used to obtain a mean value for the bacterial content of blood and tissues of experimental and control animals for each set of conditions. The most marked effect noted was the sharp increase in pneumococci in the liver of splenectomized guinea pigs 4 hours after inoculation as compared to normal animals. This increase was not associated with an appreciable increase in the bacteremia. Splenectomy of rats resulted in a much smaller increase in pneumococci in the liver than was noted in guinea pigs. Efforts at blocking the reticulo-endothelial system either by intracardial injection of 1 ml of 10% saccharated iron or intraperitoneal injection of 5 ml of the colloid had relatively little effect on the fate of injected pneumococci in either the rat or the guinea pig.

Early pathology of D. pneumoniae *type I in rats and guinea pigs.*—Animals were inoculated intracardially with about 10^8 pneumococci. At 8, 16, and 24 hours after inoculation, guinea pigs and rats were sacrificed, tissues placed in 10% formalin, and sent to the Department of Pathology of the University of Michigan for examination. Dr. Charles C. Congdon of that department reported:

> "It seems evident, even in this small series of animals, that the guinea pig and the rat are different histologically in their reaction to the organism. The difference, however, is quantitative rather than qualitative. The retrogressive changes, such as cloudy swelling and simple necrosis, are more prominent in the rat then in the guinea pig.
>
> "The defensive reaction in the guinea pig was generally limited to purulent inflammation, whereas in the rat this progressed to small foci of suppuration and microscopic abscess formation."

SUMMARY

In this general orientation study the effect of the selected pathogens on a resistant and susceptible host in terms of producing a fatal infection, the relative ability of these two hosts, the guinea pig and rat, to clear the pneumococcus from the blood stream, and from certain tissues have been determined. More detailed studies on specific defense mechanisms have been made and will be reported in other papers.

COMPARATIVE STUDY OF HOST RESISTANCE OF GUINEA PIGS AND RATS

II. EFFECT OF GUINEA PIG AND RAT SERUM ON *DIPLOCOCCUS PNEUMONIAE* TYPE I

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As part of a comparative study of defense mechanisms of the rat and the guinea pig, animals varying markedly and in an opposite sense in their resistance to *Diplococcus pneumoniae* and *Bacillus anthracis*,[1] an investigation has been made of the relative effects of the rat versus guinea pig blood or serum on *D. pneumoniae*.

GENERAL METHODS AND MATERIALS

Genetically undefined guinea pigs, selected only on a basis of health and weight (about 300 g. average), were used throughout the work.

The Sprague-Dawley strain of rats was used. No selection was made for sex; the average weight was about 150 to 200 g.

The strain of *D. pneumoniae* type I used as the pathogen for this work was maintained in the logarithmic phase of growth and at a high degree of virulence.[1]

All estimation of organisms in fluids are based on viable counts in poured plates. Brain heart infusion agar with 3 to 5% freshly added human blood was used for plating.

I. Growth of the pneumococcus in normal guinea pig and white rat blood

These tests represent the initial experiment in this investigation. They were undertaken to determine whether a difference could be detected in the growth of pneumococci in the blood from a resistant and a susceptible host species. In this series a single dilution of the pneumococci was inoculated into 3 ml samples of the blood from each species. All blood samples were run in duplicate. The normal bloods were compared to blood aliquots heated at 55 C for 1 hour before incubation with 0.1 ml of 1×10^{-5} dilution of the culture in gelation saline. All tubes were incubated at 37 C. Immediatey after inoculation 0.1 ml samples were withdrawn and plated for counting. Similar samples were withdrawn after 2, 4, and 6-hour incubation. Suitable decimal dilutions of the samples were made to insure obtaining countable plates. All plates were counted after 24-hour incubation. The averaged results of tests on 16 individual animals of each of the test species are presented in table 1.

It was shown that while the blood of neither of the test species was capable of killing the small inoculum, the blood of the guinea pig exerted a definite inhibitory effect on the growth of the organism. The ratio of the differences between the means and the standard error of the means of the values for inhibitory effect of the blood of the two species was 23. Since a value of 2 can be considered significant, this is clearly a significant difference, not a chance relationship.[2]

II. Growth of the pneumococcus in normal guinea pig and white rat serum

These tests were undertaken to obtain information as to the activity of the

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1. Nungester, W. J., Moore, V., Mika, L. and Summers, P. W. 1955, Comparative study of host resistance of guinea pigs and rats. I. Virulence of *Diplococcus pneumoniae* and *Bacillus anthracis* for guinea pigs and rats. J. Infect. Dis. 96: 227–231.

2. Hill, B. 1948, Principles of Medical Statistics, Edinburgh, R. and H. Clark, Ltd.

TABLE 1.—*The growth of pneumococcus in defibrinated blood of the rat and guinea pig.*

Species	Treatment	Population per ml serum at			
		5 minutes	2 hours	4 hours	6 hours
Rat	None	2.3×10^3	17.6×10^3	58×10^3	54.4×10^4
Population change*		1	8	252	2,365
Guinea Pig	None	2.7×10^3	5.4×10^3	5×10^3	1.7×10^4
Population change		1	2	19	63
Rat	55 C 1 hour	1.3×10^3	16.8×10^3	90×10^3	13.0×10^6
Population change		1	13	692	10,000
Guinea Pig	55 C 1 hour	2.8×10^3	7.3×10^3	22×10^3	86.0×10^3
Population change		1	3	75	307

* Population change equals $\frac{\text{observed population}}{\text{initial population}}$.

serums of the two species against pneumococci. The procedures followed were the same as those previously used for whole blood. It was observed that serum from the host species was more inhibitory than blood. The species difference in activity observed in the tests of blood was also present in the serum.

On the basis of the observation that a similar activity could be observed in the serum, it was immediately made the object of investigation. The removal of the cellular elements offered many advantages in ease of manipulation in the tests.

In order to resolve the question of inadequate nutrition as a possible cause of the observed difference in growth of pneumococci in the serum of the two test species, a series of experiments was undertaken using massive inoculation, long periods of culture, and dilution of serum. Dilutions of serum with a non-nutrient diluent offered the most promising evidence in the nutrition investigation. The serum dilutions were prepared as decimal dilutions in saline. Two dilutions of type I pneumococcus from 6-hour culture were inoculated. Population estimates were made at the time of inoculation and after 24-hour incubation.

The results indicated that dilution of the serum of both species permitted an increase in growth after some critical dilution had been reached. Higher dilution of the serums were unable to support growth. Since greater growth was obtained in diluted serum of both species than in the undiluted, it was concluded that relative nutritive value was not the critical factor affecting growth of pneumococci.

III. The activity of normal serum components in pneumococcidal action

Complement: Determinations made here indicated that the hemolytic complement titer (using rabbit anti-sheep cell serum and 50 % hemolysis units) of normal guinea pig serum was approximately 5.5 times that of normal rat serum. The figures were derived from values determined on eight pooled samples of each species. This indicated that the resistant species, the guinea pig, also possessed the higher complement titer. Individual samples did not demonstrate a fixed relation between complement titer and inhibitory power.

Normal concentrations of complement were increased by addition of complement fractions prepared by the dilution method of Ferrata.[3] The fractions of guinea pig and rat serum thus obtained were tested by adding them to fresh serum samples in an attempt to augment existing bactericidal activity. All samples were compared to normal non-reinforced serum controls. None of

3. Ferrata, A. 1907, Die Unwirksamkeit der komplexen Hamolysine in salzfrein Losungen und ihre Ursache. Berlin Klin. Wchnschr. **44**: 366–368.

the complement fractions obtained from the serum of either species showed any ability to increase the normal inhibitory power of the serums to which they were added.

Within a few minutes after heating at 56 C. serum loses complementary activity because of the destruction of C'_1 and C'_2. C'_3 and C'_4 are more resistant, but if heating is continued for 30 to 40 minutes, most of the C'_3 and C'_4 activity may be lost as well.[4] The effect of heating for varied periods of time and at various temperatures on the inhibitory activity of normal serums was investigated. In all cases the inhibitory power of the serum from either rat or guinea pig was found to increase proportionally to the length of time of heating or the temperature of storage up to 50 C. used in these tests.

It would seem that, while the complementary activity and the inhibitory power for pneumococcus are both greater in the guinea pig than in the rat, the simultaneous occurrence is not a cause and effect relationship, since destruction of the complementary activity does not adversely affect the inhibitory power.

Normal antibodies.—All attempts to detect the presence of antipneumococcal antibody in the serum of normal guinea pigs and rats have failed. The agglutination technique was employed, with both macroscopic and microscopic observations. No quelling reaction has been observed with serum from normal individuals of either species.

Serum fractions.—Several fractionation techniques, salt, ethanol, and distilled water were used in an attempt to associate the antipneumococcal activity of the serum of the guinea pig with a definite component. None of the fractions of serum tested consistently fortified the existing bactericidal power of the serum against pneumococcus. The most frequent observation obtained was that addition of a fraction lowered the bactericidal activity. This was particularly true of the protein fractions.

4. Kabat, E. A. and Mayer, M. M. 1948, Experimental Immunochemistry, Springfield, Ill., Thomas.

IV. The effect of some physical and chemical manipulations on the Growth of pneumoccocci in rat rat and guinea pig serums

In the course of experimentation on the effect of agitation on the bactericidal activity of blood, it was noted that agitated serum increased markedly in bactericidal activity. The cause of this change was investigated.

Pooled fresh rat and guinea pig serums were prepared and distributed in 1 ml quantities to test tubes. An immediate determination of the bactericidal activity, with the test tubes stationary, was made on one series from each species. A second series was agitated for 24 hours at 37 C before testing. After agitation, pH values were determined and adjusted to physiological range by addition of 1 *N* HCl. A bactericidal test was then made with the tubes stationary. The results presented in table 2 indicate that a basic shift in pH

TABLE 2.—*The effect of agitation in air for 24 hours on the pH and pneumococcidal activity of rat and guinea pig serum: pneumococcidal power after neutralizing agitated serum.*

Species	Shaken	pH shift on shaking	pH adjusted	Population per ml at	
				0 hours	24 hours
Rat	−			9×10^6	1×10^9
	−			9×10^6	0
	−			9×10^6	0
Rat	+	7.7–8.6	7.2	7×10^6	2×10^9
	+	7.7–8.6	7.2	7×10^6	1×10^7
	+	7.7–8.6	7.2	7×10^6	3×10^9
Guinea pig	−			9×10^6	2×10^9
	−			9×10^6	0
	−			9×10^6	0
Guinea pig	+	7.6–8.1	7.3	7×10^6	4×10^9
	+	7.6–8.1	7.3	7×10^6	40
	+	7.6–8.1	7.3	7×10^6	0

occurred on agitation and that neutralization of the serum removed the bactericidal power of the rat serum and reduced that of the guinea pig.

When the serum buffer capacity was increased by the addition of 0.3 ml of 0.15 *M* phosphate buffer (pH 7.2) to 1.0 ml of serum, it was found that the marked basic shift in pH was prevented and the bactericidal power did not increase on aeration.

When fresh serum was made basic by addition of 1 *N* NaOH, it was found to be equally toxic for pneumococci. It was considered that the increased toxicity was due to the sensitivity of the pneumococci to the induced alkalinity of the serum.[5]

Observations of 1.0 ml quantities of serum incubated at 37 C indicated that the serum of both species can reach pH of 8.3 in 3 hours and 8.9 to 9.0 in 24 hours. When the serums were placed in a partial vacuum (5 cm Hg), a shift from the normal value of fresh serum to 8.2 to 8.3 was achieved in 15 minutes. CO_2 was considered the component most likely to be lost from the serum.

Tests were made to determine the change in pH values when gaseous exchange was minimized. Blood was collected and the serum separated under sterile mineral oil. The serum was distributed in 1.0 ml amounts under mineral oil seal and the pH values determined after time intervals. After 24 hours under mineral oil, the pH value for the rat serum was 7.05 and for the guinea pig serum, 7.15. It is apparent that the mineral oil had prevented the basic shift.

According to Van Slyke, the CO_2 tension of alveolar air for normal resting adult humans is 4.7 to 6.8%. Serum equilibrated with a 5% concentration of CO_2 might approximate physiological concentration for arterial blood, of that component. When serum was distributed as before and held in a closed jar with a 5% CO_2 and 95% N_2 atmosphere, the pH shifted only slightly to 7.8 after 24 hours.

Evaluation of the bactericidal activity of the serums of the test species under mineral oil and under CO_2-N_2 atmosphere showed the pneumococcidal activity to be lessened. Representative data are shown in table 3. It can be seen from these data that under the controlled atmosphere conditions rat serum did not destory a minimum inoc-

5. Dernby, K. G. and Avery, O. T. 1918, The optimum hydrogen ion concentration for the growth of pneumococcus. J. Exper. Med. **28**: 345–357.

TABLE 3.—*The effect of controlled atmospheres on the pneumococcidal activity of rat and guinea pig serum.*

Species	Atmosphere	Population per ml at		
		0 hours	6 hours	24 hours
Rat	Oil seal	12×10^8	1×10^8	1×10^8
	Oil seal	12×10^6	1×10^7	1×10^6
	Oil seal	12×10^3	1.5×10^6	1×10^6
	Oil seal	12	2.3×10^6	1×10^6
Guinea pig	Oil seal	12×10^8	1×10^7	1.1×10^6
	Oil seal	12×10^6	1.7×10^6	9.4×10^3
	Oil seal	12×10^3	40	0
	Oil seal	12	0	0
Rat	5% CO_2-95% N_2	15×10^8	1×10^7	1×10^8
	5% CO_2-95% N_2	15×10^6	1×10^7	1×10^8
	5% CO_2-95% N_2	15×10^3	5.8×10^6	1×10^8
	5% CO_2-95% N_2	15	1.9×10^6	1×10^8
Guinea pig	5% CO_2-95% N_2	15×10^8	1×10^7	4×10^6
	5% CO_2-95% N_2	15×10^6	1.5×10^6	1×10^6
	5% CO_2-95% N_2	15×10^3	5.6×10^6	2.3×10^6
	5% CO_2-95% N_2	15	40	0

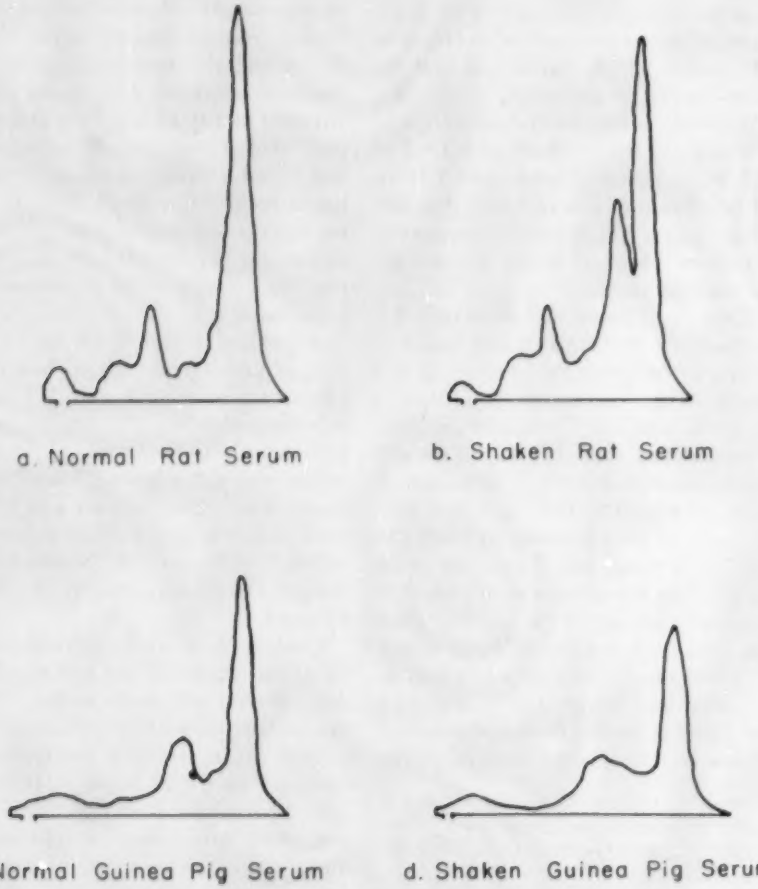

FIG. 1.—Electrophoretic patterns of normal rat and guinea pig serum proteins before and after 24-hour aeration by shaking. Veronal buffer, pH 8.6. a. Migration time 180 min, potential gradient 9.3 v/cm; b. 180 min, 9.0 v/cm; c. 200 min, 7.7 v/cm; d. 220 min, 7.9 v/cm. Descending arms.

ulum. Although the pneumococcidal power of guinea pig serum was reduced in the anaerobic tests, the number of viable organisms was still reduced in 24 hours. Under 5% CO_2, a stasis in the 1×10^3 inoculum is effected with a resulting decline in numbers.

With an addition of O_2 to the CO_2-N_2 atmosphere it was observed that the pH readings shifted to basic values, indicating that factors other than the breakdown of the bicarbonate-carbonate buffer system were involved. When an atmosphere of 76% N_2, 4% CO_2, and 20% O_2 was used, pH values of 8.0 were reached in both rat and guinea pig serums in 24 hours at 37 C. Employing an atmosphere of 9.5% CO_2, 44% O_2, and 66.5% N_2, a similar basic shift was observed. Even this higher concentration of CO_2 was unable to maintain normal pH values in the presence of O_2.

Electrophoretic analysis using a Tiselius apparatus revealed that a redistribution of the protein components of rat serum on aeration was reduced

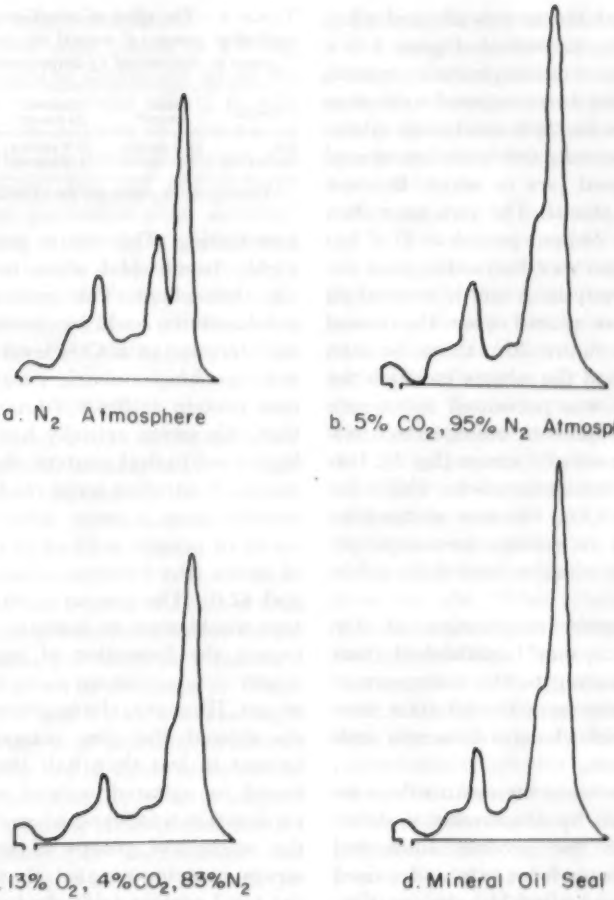

FIG. 2.—Electrophoretic patterns of normal rat serum exposed for 24 hours to varying atmospheres. Veronal buffer, pH 8.6; migration time 180 min. Potential gradient: a. 9.0 v/cm; b. 6.9 v/cm; c. 6.7 v/cm; d. 7.1 v/cm. Descending arms.

when loss of CO_2 was minimized. The patterns obtained from rat and guinea pig serums before and after aeration are shown in figure 1. These serums were run in veronal buffer at pH 8.6.

The new peak shown on the treated rat serum sample (fig. 1b) comprises 28.7% of the total protein; the albumin fraction of the same sample is 45.6%. The combined concentration of the albumin and the new components is 74.3%. Planimetry of the pattern obtained from normal serum shows that the albumin component is 71.5% of the total protein represented. This suggests that the new component developing on agitation emerges from the albumin fraction. No significant change in the distribution of the protein fractions occurred in agitated guinea pig serum.

When the increase in bactericidal property of the serums on agitation was shown to be related to the loss of CO_2 from the serum, further electrophoretic patterns were obtained to determine whether the formation of the new com-

ponent in rat serum was affected when CO_2 loss was prevented. Figure 2 is a reproduction of electrophoretic patterns obtained from serum exposed to various atmospheres for 24 hours before analysis. The selected gases were introduced into evacuated jars in which the test tubes were placed. The jars were then stored for a 24-hour period at 37 C before the serum was dialyzed against the buffer for analysis. A sterile mineral oil covering was placed over the serum analyzed in figure 2d. It can be seen that in none of the serums in which the loss of CO_2 was prevented did a seccondary component emerge as completely as in aerated serum (fig. 1). Under the N_2 atmosphere into which the serum loses CO_2, the new component can be seen to emerge as completely as when the samples were agitated in air.

The generous cooperation of Dr. Makio Murayama* established that conditions leading to the emergence of the component from the albumin were associated with changes in serum sulfhydryl content.

Table 4 presents the mean values for data obtained by amperometric determination of the protein sulfhydryl groups of serum from rats and guinea pigs before and after 24-hour aeration.

It can be seen from the data presented that aeration which produced a basic shift in pH values of the serum also produced a change in the protein sulfhydryl groups. There is a twentyfold difference in the drop in titrable sulfhydryl groups between the serums of the two species. The fact that the drop in titrable sulfhydryl groups is observed in rat serum which also developed the new component suggests that the two events are related.

An examination was made of the serum of rats starved for 72 hours before testing. This serum proved to be highly bactericidal when tested in an air atmosphere. The marked bactericidal activity could be removed by the maintenance of a CO_2 level of 5% by volume. Amperometric titration of serum protein sulfhydryl groups showed that this serum actually had a slightly higher sulfhydryl content than normal serum. A titration series made on seven animals gave a mean value of 41.5 μ moles of protein sulfhydryl per 100 ml of serum (the extreme values were 41.0 and 42.0). The greater sulfhydryl content would seem to indicate one might expect the formation of more of the slower component on agitation of this serum. However, electrophoretic analysis showed the new component was formed in less than half the quantity found in agitated normal serum. An explanation was found when titration of the sulfhydryl groups of starved rat serum protein revealed that although the total protein sulfhydryl groups had increased over the content of normal serum, there was a marked qualitative difference in the reactivity of the sulfhydryl groups of the starved rat serum. The value for the sulfhydryl groups before aeration was determined as 40.0 μ moles per 100 ml of serum. On aeration this value was observed to drop to 35.0 μ moles per 100 ml, a difference of only 5.0 μ moles, as compared to the value of 16.4 μ moles difference obtained on aeration of normal rat serum.

The addition of sulfhydryl-containing compounds to the test serums indicated that these compounds were capable of lowering the bactericidal power when

TABLE 4.—*The effect of aeration on the protein sulfhydryl groups of normal rat and guinea pig serum as determined by amperometric tiration.*

Species	Fresh serum*	Aerated 24 hours*	Difference*
Rat	37.2 μmoles	20.8 μmoles	16.4 μmoles
Guinea pig	32.2 μmoles	31.4 μmoles	0.8 μmoles

* Values given as μmoles per 100 ml serum.

* Harper Hospital, Detroit, Michigan.

added in sufficient quantities.

Thioglycollic acid added in 1.0 mg per ml amounts of normal rat serum removed the bactericidal effect. It was found that the addition of as much as 22 mg of the compound to guinea pig serum lowered, but did not entirely remove, the pneumococcidal activity. The addition of 10 mg of glutathione per ml of rat serum was necessary to remove the bactericidal activity. A similar quantity added to guinea pig serum did not markedly affect the activity.

DISCUSSION

In inoculated serum incubated in air the tendency to become basic through loss of CO_2 is opposed by the acid production of the pneumococcus. When sufficient organisms are present the serum becomes slightly acidic. With fewer organisms present the loss of CO_2 will shift the pH to the alkaline range. The optimum pH for type I pneumococcus is 7.8.[3,4] The extremes are given as 7.0 to 7.2 and 8.2 to 8.3. This indicates that the pneumococcus is limited in growth by a pH value achieved by sterile serum in air at 37 C in slightly less than 3 hours. Acid production by the pneumococcal metabolism can counterbalance this shift in some instances. It is likely, then, that when serum is tested in air a higher bactericidal titer will be obtained than when CO_2 loss is limited. Clearly this could be misleading in in vitro tests of bactericidal activities in serum systems.

An additional factor must be considered in interpretation of growth under conditions of varied CO_2 concentrations: the stimulating effect of that gas. Kempner and Schlayer[6] have shown that 0.1 volume percent permitted growth, but the growth did not become logarithmic for 21 hours.

Tests of the bactericidal property of serum under enriched CO_2 atmospheres showed that all activity could be removed from rat serum. Under the same conditons guinea pig serum destroyed approximately 1000 pneumococci per ml in 24 hours.

Oxygen, even in quantities up to 44% of an atmosphere, produced little change in batericidal activity of guinea pig serum. At the higher concentrations, however, even in the presence of CO_2 concentrations shown to be capable of maintaining stable pH values in the absence of O_2, a basic shift occurred. The mechanism of this basic change under the influence of O_2 is not apparent.

Dubos[7] has demonstrated that in the oxidized form methylene blue and indophenol are bacteriostatic for pneumococci, while indigo and certain other dyes are not. When these inhibiting dyes were present in reduced form in a medium capable of maintaining them in that condition, they were no longer toxic. Certain commercial peptones were also shown by Dubos[8] to possess bacteriostatic activity gainst pneumococci when incorporated into an otherwise adequate medium. These peptone preparations were active when oxidized, inactive when reduced. Thioglycollic acid added to the oxidized peptone medium was able to overcome the bacteriostatic activity, permitting a small inoculum to grow.

In view of the fact that bacteria are known to exert a strong reducing force during active growth, relative oxidation-reduction capacities of the organisms inoculated and the oxidation-reduction system of the medium play a most im-

6. Kempner, W. and Schlayer, O. 1942, The effect of carbon dioxide on the growth rate of the pneumococcus. J. Bact. **43**: 387–396.

7. Dubos, R. J. 1929, The relation of the bacteriostatic action of certain dyes of oxidation-reduction processes. J. Exper. Med. **49**: 575–592.

8. Dubos, R. J. 1930, The bacteriostatic action of certain components of commercial peptones as affected by conditions of oxidation and reduction. J. Exper. Med. **52**: 331–345.

portant role. A larger number of organisms inoculated into a medium with a strong oxidizing potential but with a weak capacity for poising might readily reduce the oxidation-reduction system of the medium, and grow well. A smaller inoculum against the same system might be expected to be held in stasis and die.

Determination of the potential developed by fresh serum of the rat and guinea pig indicated that the later is electropositive compared to the rat serum. The difference was 52 to 57 millivolts between the serums freshly exposed to air. A consistently greater reducing potential in the rat serum was obtained throughout 4 hours of exposure of the serum to air. The system was not in equilibrium during the observation period. This factor and the complexity of the serum make interpretation of the observed potentials difficult. However, since the two systems were measured by the same technique under experimentally identical circumstances, it is felt that these relative values may be considered valid. The relatively greater reducing potential observed in the rat serum may be responsible for the growth of an inoculum which cannot overcome the greater oxidizing potential in guinea pig serum.

Sulfhydryl containing compounds, such as glutathione and thioglycollic acid, added to serum, acted to remove the bactericidal activity even when tested in air. Smaller quantities of the reducing compounds were necessary to promote growth in the rat than in the guinea pig serum. The qualitative difference demonstrated in the sulfhydryl groups of rat serum compared to guinea pig serum has been in their lability. The twentyfold difference in reactivity is probably related to the lower oxidation potential of rat serum.

Schoenbach et al[9] have recently shown that in a variety of abnormal conditions, including infection, the sulfhydrul content of human serum decreases. In 13 samples of serum from infected patients there was on the average 1.43 mg less of cysteine equivalent per 100 ml than in normal serum. This observation might lend itself to an explanation of the increased bactericidal action of human serum drawn at the time of acute infection, as noted by Tillet.[10] He surmised that "the element in serum responsible for the death of streptococci is active in oxidized form but seriously impared when reduced"[11]. Together with Stock,[12] he observed that an aerobic conditions lessen the basic shift in human serum and also lower the bactericidal power. It is possible that the lower sulfhydrul content of the acute phase serum accounts for its activity compared to normal serum under conditions in which toxic pH changes do not occur.

Electrophoretic analysis of rat and guinea pig serum after aeration by gentle shaking showed the presence of a component not found in fresh normal rat serum. No such change was observed in the guinea pig serum. Planimetric analysis of the distribution of the serum proteins suggests that the in-

9. Schoenbach, E. B., Armistead, E. B. and Weissman, N. 1950. The sulfhydryl content of normal and abnormal human serums. Proc. soc. Exper. Biol. & Med. **73**: 44–46.

10. Tillet, W. S. 1937, The bactericidal action of human serum on hemolytic streptococci. I. Observations made with serum from patients with acute infection and normal individuals. J. Exper. Med. **65**: 147–161.

11. Tillet, W. S. 1937, The bactericidal action of human serum on hemolytic streptococci. II. Factors which influence the phenomenon in vitro. J. Exper. Med. **65**: 163–176.

12. Tillet, W. S. and Stock, C. C. 1937. The bactericidal action of human serum on hemolytic streptococci. III. Studies concerning (1) significance of hydrogen ion concentration in relation to streptococcidal action of serum, and (2) the effect of reducing agents on the phenomenon. J. Exper. Med. **66**: 617–637.

duced component emerges from the albumin, since these two components vary in an inverse relationship.

Moore et al[13] observed changes in rat serum from hypophysectomized animals compared to normals. The albumin-globulin ratio of the serum determined chemically and electrophoretically was observed to be lower in the treated animals. These latter specimens showed an α globulin component lacking in the normal serum.

Moore[14] in analysis of adult rat serum in barbiturate buffer shows a distribution of 58% and 20% for ablumin and "α_1 globulin" (components 1 and 2). The distribution of components in immature rats serum under the same condition was 72% ,and 6%, respectively. It is possible that the distribution of components in these patterns has been influenced by conditions of storage before analysis.

Roberts and White,[15] studying the origin of serum proteins, made Tiselius patterns of normal and treated serum from Sprague-Dawley rats. These workers observed that rat serum incubated 3 hours with minced rat liver (200 mg/2 ml serum) underwent a change in its serum components. During the period of incubation an atmosphere of 95% O_2 and 5% CO_2 was employed, except in a single instance in which 100% N_2 was used. The greatest component changes were observed in the lowering of concentration of albumin and increase in the "α_1 globulin." The toal concentration of ablumin plus "α_1 globulin" in the untreated sample was 71.0% of the total and in the treated sample 69.5% of the total. The authors noted that "the processes involved in the loss of albumin and gain in "α_1 globulin" occurred under anaerobic as well as aerobic conditions and might be causally related."

The results of the present study are in complete agreement with this causal relationship. However, the critical factor appears to be the CO_2 tension with its related hydrogen ion effects, rather than O_2 tension alone.

Observations made here indicate that the "α_1 globulin" component obtained by Roberts and White in normal and treated serums can be prevented from forming in the normal samples if equilibration with air is prevented by mineral oil seal during preparation.

In view of the fact that sterile serum without added tissue has been shown to devlop a new component resembling the "α_1 globulin," the addition of minced liver cannot be considered the determining fact of in the "liberation" of this component as suggested by Roberts and White.

SUMMARY

The effect of blood and serum from normal guinea pigs and white rats on the growth of type I pneumococci in vitro has been determined.

Both blood and serum from the resistant host (guinea pig) were shown to be more pneumococcidal than that from white rats. Part of the activity in the in vitro test can be attributed to toxic pH changes brought about by the loss of CO_2 from the serum. Evidence is presented relating the pneumococcidal action in the absence of toxic pH change to a higher oxidation-reduction potential in guinea pig serum.

A controllable variation in the distribution of rat serum proteins fractions was observed by electrophoretic analysis.

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THE EFFECT OF X IRRADIATION ON IMMUNITY OF THE MOUSE TO *TRYPANOSOMA DUTTONI*

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Studies on the effects of X irradiation on immunity have been most recently reviewed by Taliaferro and Taliaferro.[1] In general, the results of these and subsequent investigations indicated that X irradiation in large doses inhibits antibody formation and increases susceptibility to infection.

In the present investigation the course of infection by *Trypanosoma duttoni*, a nonpathogenic hemoflagellate of the mouse, was followed in irradiated mice. This parasite offers several advantages in the study of immunity. Among these are: (1) The parasite is large enough for morphological study. (2) The parasite is limited to the blood stream; therefore, changes in the parasitic population may be observed daily without sacrificing the host. (3) The extent of reproductive activity of the parasite may be followed by daily examination of blood smears for division forms. The last gives a measure of the rate of reproduction irrespective of the number of organisms killed. Measurement of the basic rate of parasite reproduction in conjunction with change in numbers of the surviving population permits a sharp distinction between reproduction-inhibiting and parasiticidal effects.

Previous work on *T. duttoni* has indicated that: (1) There is an innate factor in normal mice which partially inhibits reproduction of the trypanosomes. This partial inhibition, which disappears as the result of splenectomy and India ink blockade,[2] is indicated by the lower percentage of division forms (a measurement of reproductive activity) in the trypanosome population in control as compared to splenectomized-blockaded animals. It is not due to a passively transferable antibody or serological principle.[3] (2) After the infection has progressed 6 to 8 days, an antibody (ablastin) is formed which further inhibits reproduction of the parasites.[3] (3) Eventually, one or more parasiticidal antibodies (lysins) are formed which kill large numbers of parasites.[3]

This investigation, outlined in a preliminary report,[4] is concerned with the study of the effects of X irradiation on the immunity of the mouse to *T. duttoni*, with special reference to the innate phase of immunity.

MATERIALS AND METHODS

The strain of *T. duttoni* used in these studies was obtained in Puerto Rico and has been maintained by Dr. W. H. Taliaferro at the University of Chicago since 1945.

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The author wishes to express his appreciation to Dr. William H. Taliaferro for his criticism and assistance in the course of this investigation.

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The mice used in all experiments were 10- to 16-week-old CF #1 females. Animals composing an experimental group were of the same age.

The experimental mice were infected intravenously with trypanosomes from donor mice with 10-day-old infections. Blood was obtained from anesthetized donor mice by cutting the brachial artery and collecting the blood from the pocket formed by the skin and thoracic wall.[5] The blood was centrifuged in 15 ml conical centrifuge tubes at a relative centrifugal force (RCF) of 150 times gravity (g) at room temperature for 25 minutes. The trypanosomes were decanted off in the supernatant plasma and centrifuged at 4 C at a RCF of 1700 g in Kolmer centrifuge tubes for 40 minutes. They were resuspended in phospate-saline solution similar to that of Krebs and Henseleit,[6] as modified by Moulder.[5] The number of trypanosomes for inoculation into experimental mice was determined from hemocytometer counts.

The infecting dose in various experiments ranged from 2 to 10×10^6 parasites. All mice in any one experiment received the same number. The course of the infection did not seem to depend on the size of the inoculum.

Trypanosome counts were made every 2 days, using the technique of Kolmer.[7] The tip of the mouse's tail was cut, and the blood was taken up in a red-cell pipette and diluted to mark with 1.5% acetic acid. Parasite counts were then made with a hemocytometer.

Blood smears were made daily, fixed in absolute methyl alcohol for 5 minutes, air-dried, and stained in 4% Giemsa stain in buffered water, pH 7.2. The level of reproductive activity of the parasites was measured in terms of percent "young" and dividing forms, according to the method of Taliaferro, Cannon, and Goodloe.[8] Fifty to 100 trypanosomes were examined consecutively and classified as "adult," "young," or dividing forms. In those smears where few parasites could be found, only 25 trypanosomes were examined. Any trypanosome 25 μ or less, measured from the tip of the flagellum to the posterior end, was classified as "young," while any above 25 μ was placed in the "adult" classification. When doubt existed as to category, the parasite was drawn with the camera lucida at 3000 magnification and measured with a calibrated pair of dividers. When division had proceeded to a point where multiple flagella were present, each potential daughter cell was counted as an individual.

All mice received subcutaneous injections of 6 mg of dihydrostreptomycin daily, beginning 2 days after irradiation and continuing until the end of the experiment. According to Miller et al.,[9] this was the method of choice for minimizing postirradiation bacteremia. This regimen of dihydrostreptomycin dosage did not affect the course of the trypanosome infection.

The mice in all experiments were irradiated in small, ventilated plastic tubes arranged in a circle on a rotating table. X-ray factors were: 250 KV, 15 ma, 0.5 mm Cu plus 3 mm Bakelite filters, HVL = 1.5 mm Cu. Dose rate was 22 r per minute as measured by a Victoreen chamber in air inside a plastic tube at the same location as the center of the mouse. Target distance was 69 cm. Total dose for all mice was 550 r. All infected animals died within 14 days after exposure to this dose of radiation.

RESULTS

Effects of X irradiation on the course of infection

All mice were infected with *T. duttoni* one day after irradiation of the experimental group.

The curves (fig. 1) of reproductive activity represent the mean daily value for 90 irradiated (X) and 81 nonirradiated (C) mice. Parasitemia curves show the mean value for 14 mice, chosen at random, in each of the two groups. Reproductive activity in the controls proceeded at a low level until the 6th day, when it decreased, owing to the formation of ablastin. The parasitemia rose slowly but never exceeded 50,000 trypanosomes per cmm in any individual

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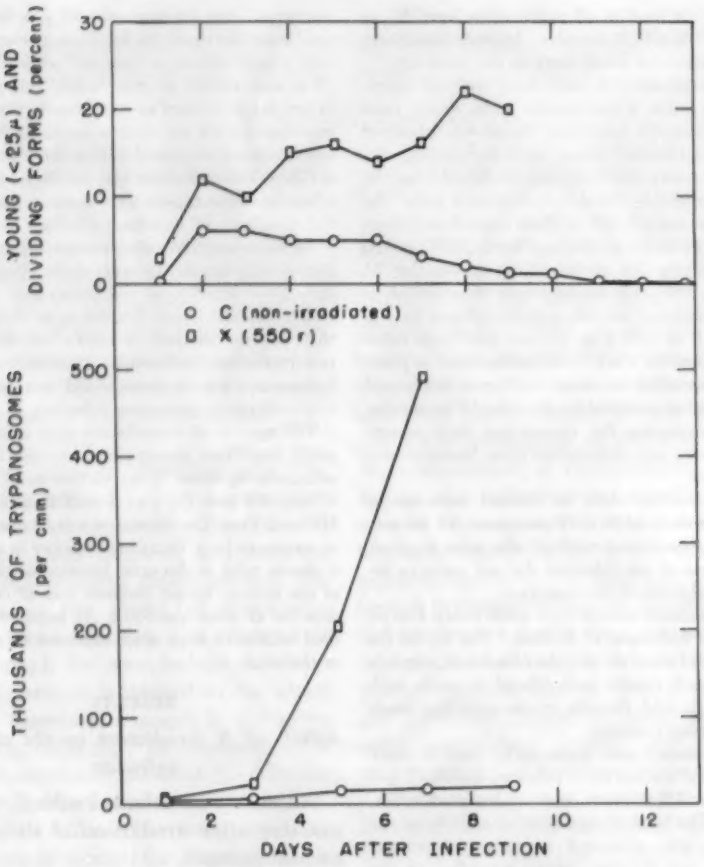

FIG. 1.—The course of *T. duttoni* infection in 81 nonirradiated (C) mice and 90 irradiated (X) mice, as shown by reproductive activity (young and dividing forms) and parasitemia (thousands of trypanosomes).

of the group. The reproductive activity and the parsitemia in irradiated animals increased rapidly and gave no indication of decreasing up to the time of death. As early as the 3rd day after infection, the parasitemia in the irradiated animals was 18.32 ± 4.53 thousand per cmm as compared to 6.86 ± 1.26 thousand per cmm for controls, with $P < 0.01$. Analysis for differences in reproductive activity was made by comparing the peak division rate in each mouse of the two groups during the first 5 days after infection. This was the period before the onset of acquired immunity could first be detected. The means were $23 \pm 1\%$ young and dividing forms for irradiated and $10 \pm 1\%$ for controls, with $P < 0.001$.

The parasitemia, although influenced by reproductive activity, was not solely dependent upon this factor. This is clearly demonstrated in figure 2. An unirradiated mouse (C59) in which the reproductive activity of the parasites was high is compared to an irradiated

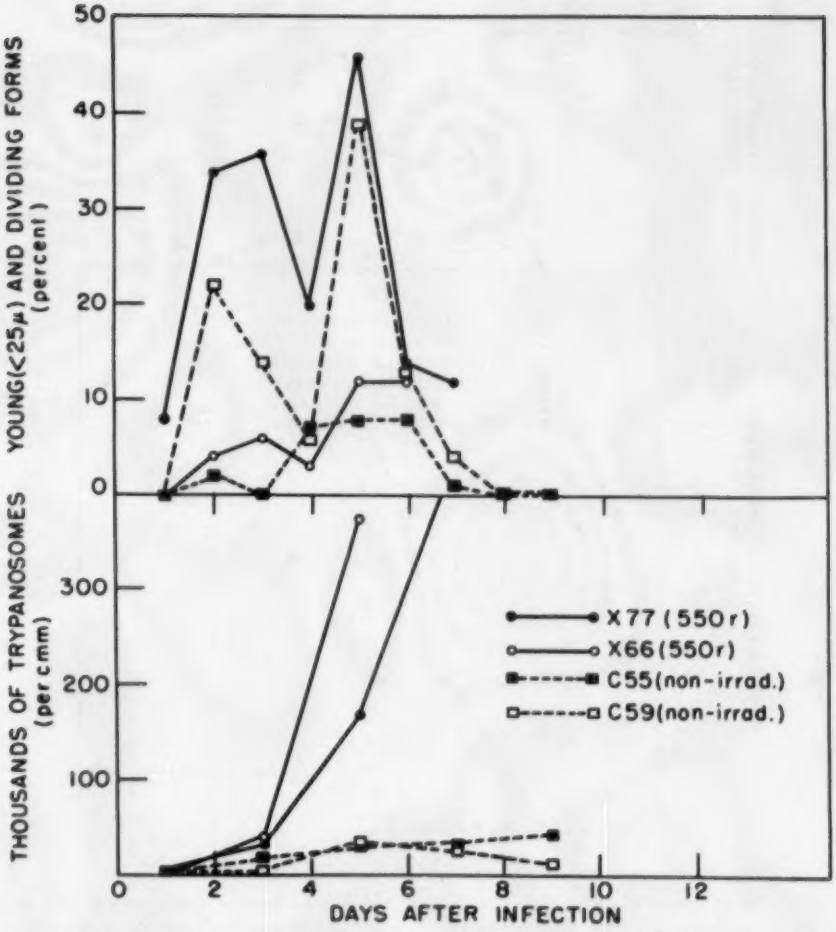

FIG. 2.—The course of *T. duttoni* infection in 2 unirradiated (C59 and C55) and 2 irradiated (X66 and X77) mice. Note the lack of correlation between reproductive activity (young and dividing forms) and parasitemia (thousands of trypanosomes).

mouse (X66) in which it was low during the first 5 days of infection and vice versa (C55 and X77). The parasitemia was of the same order of magnitude in the mice receiving the same treatment. These examples suggest that in normal mice independent trypanocidal and reproduction-inhibiting factors are present which are inhibited or destroyed by irradiation at this dose level.

Effects of X irradiation at various times before infection

Groups of mice were irradiated at 1 (X), 4 (4X), 8 (8X), 15 (15X), or 22 (22X) days before infection; and one group (C) was unirradiated. The number of animals in each group was 20, 20, 16, 5, 7, and 20, respectively.

The high level of reproductive activity and parasitemia observed in the

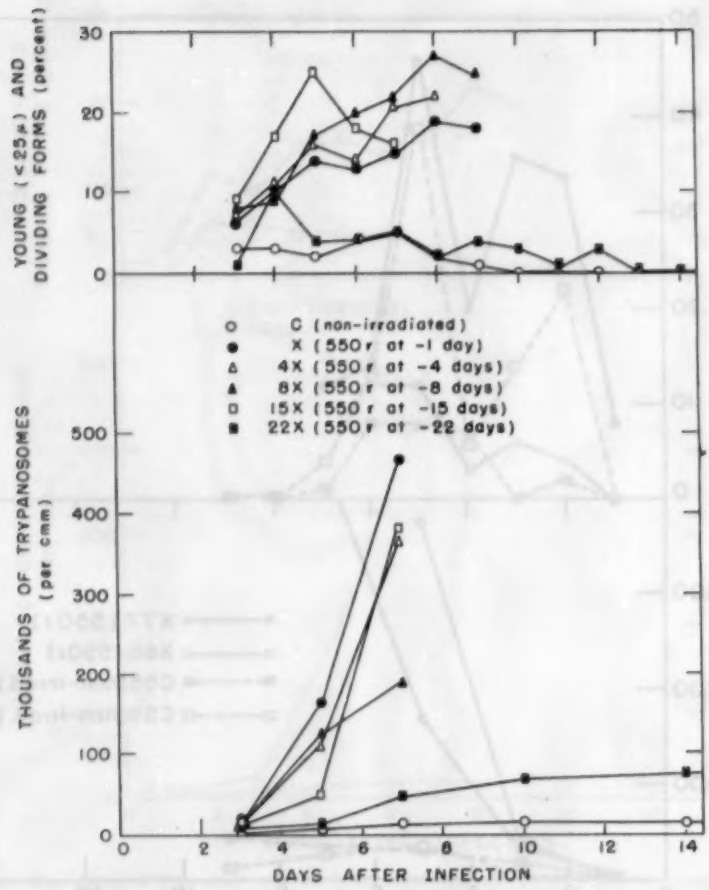

FIG. 3.—Effects of X irradiation on the course of *T. duttoni* infection in mice irradiated at 1, 4, 8, 15, or 22 days before infection, as shown by parasitemia (thousands of trypanosomes) and reproductive activity (young and dividing forms). Each curve represents the mean values for each group.

groups X, 4X, 8X, and 15X (fig. 3) indicated that up to 15 days after irradiation recovery of innate immunity was not apparent. The level of reproductive activity and the parasitemia of group 22X, intermediate between the other irradiated and control groups, suggested that a partial recovery of resistance had occurred. That recovery was only partial is further reflected in the 4- to 6-day delay in the onset of acquired immunity.

Effects of X irradiation at various times after infection

The effect of irradiation at different stages during the course of infection was investigated next. Thirty mice were irradiated at ½ hour after infection (1X); 10 mice were exposed 2 days after infection (2IX), when antigen absorption should have been in progress for some time; 15 mice were irradiated 4 days after infection (4IX), when the parasites were actively reproducing; 5 mice

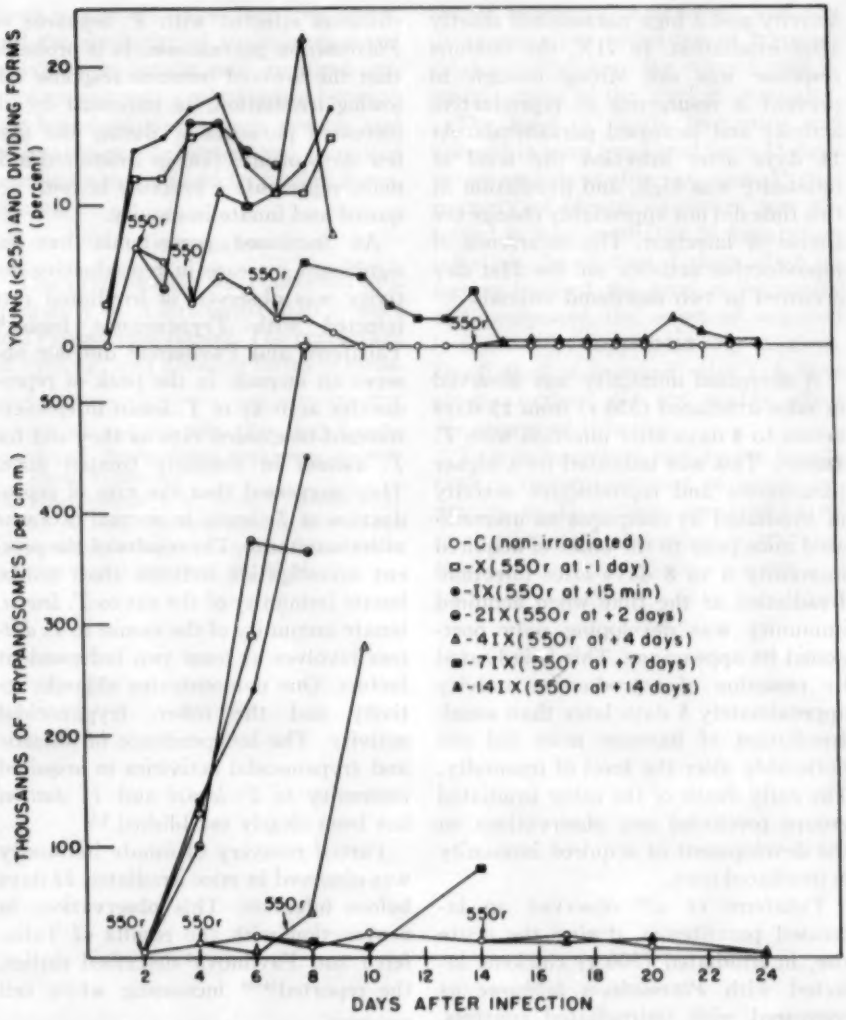

FIG. 4.—Effects of X irradiation on the course of *T. duttoni* infection in mice irradiated 15 minutes, 2 days, 4 days, 7 days, or 14 days after infection, as shown by parasitemia (thousands of trypanosomes) and reproductive activity (young and dividing forms). Each curve represents the mean values for the group.

were irradiated 7 days after infection (7IX), when acquired immunity was becoming noticeably effective; and 5 mice were irradiated 14 days after infection (14IX), when acquired immunity had already developed. Two control groups of 30 mice each consisted of a nonirradiated group (C) and a group irradiated 1 day before infection (X).

Groups X, 1X, 2IX, and 4IX (fig. 4), which were irradiated before the development of acquired immunity, showed a high level of reproductive

activity and a high parasitemia shortly after irradiation. In 71X, the immune response was not strong enough to prevent a resurgence of reproductive activity and increased parasitemia. At 14 days after infection the level of immunity was high, and irradiation at this time did not appreciably change the course of infection. The recurrence of reproductive activity on the 21st day occurred in two moribund animals.

DISCUSSION

A decreased immunity was observed in mice irradiated (550 r) from 15 days before to 4 days after infection with *T. duttoni*. This was indicated by a higher parasitemia and reproductive activity in irradiated as compared to unirradiated mice prior to the onset of acquired immunity 6 to 8 days after infection. Irradiation at the time when acquired immunity was developing only postponed its appearance. This is indicated by cessation of reproductive activity approximately 5 days later than usual. Irradiation of immune mice did not noticeably alter the level of immunity. The early death of the other irradiated groups precluded any observations on the development of acquired immunity in irradiated mice.

Taliaferro et al[10] observed an increased parasitemia, during the acute rise, in irradiated (500 r) chickens infected with *Plasmodium lophurae* as compared with unirradiated controls. Bennison and Coatney[11] obtained the same results with irradiated (400 r) chickens infected with *P. lophurae* or *Plasmodium gallinaceum*. It is probable that the lowered immune response following irradiation, as indicated by an increased parasitemia during the first few days of infection in irradiated animals, represents a lowering of both acquired and innate immunity.

An increased parasitemia but no significant increase in reproductive activity was observed in irradiated rats infected with *Trypanosoma lewisi*.[12] Taliaferro and Pavlinova[2] did not observe an increase in the peak of reproductive activity of *T. lewisi* in splenectomized-blockaded rats as they did for *T. duttoni* in similarly treated mice. They suggested that the rate of reproduction of *T. lewisi* in normal rats was at its maximum. The results of the present investigation indicate that, unlike innate immunity of the rat to *T. lewisi*, innate immunity of the mouse to *T. duttoni* involves at least two independent factors. One demonstrates ablastic activity and the other, trypanocidal activity. The independence of ablastic and trypanocidal activities in acquired immunity to *T. lewisi* and *T. duttoni* has been clearly established.[2,13]

Partial recovery of innate immunity was observed in mice irradiated 22 days before infection. This observation, in conjunction with the results of Taliaferro and Pavlinova[2] described earlier, the reported[14–16] increasing white cell

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count in mice 20 days after irradiation, and the similarity of antitrypanosomal activities in innate and acquired immunity, suggests a relationship between the lymphoid-macrophage system and maintenance of innate and acquired immunity that should be further investigated.

SUMMARY

This investigation was concerned with immunity of X-irradiated (550 r) mice to *Trypanosoma duttoni*. The percentage of "young" and dividing forms (a measure of reproductive activity) and the parasitemia (an indication of trypanocidal activity) were the criteria used in measurement of the level of immunity.

The level of innate immunity was lowered in mice irradiated from 15 days before to 4 days after infection. Partial recovery of innate immunity was observed in mice irradiated 22 days before infection. Irradiation 7 days after infection lowered the level of immunity and postponed the onset of acquired immunity by approximately 5 days. Irradiation of immune mice (14 days after infection) did not visibly affect the course of infection.

Two independent factors are important in the maintenance of innate immunity to *T. duttoni;* one inhibits reproduction and the other is trypanocidal.

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THE EFFECTS OF β-PROPIOLACTONE ON THE METABOLISM OF *CANDIDA ALBICANS*

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Concurrently with increased use of the newer broad-spectrum antibiotics there appears to be an increased incidence of fungus infections, some of them fatal, attributable to *Candida albicans*.[1] These secondary infections seem to occur most often upon oral administration of a given drug, and although the digestive tract is the site frequently affected, the mycosis may be general and systemic.[2] The situation is complicated by the fact that *C. albicans* is particularly resistant to therapy.[3,4]

The metabolism of Candida has been investigated by several workers. Lamb and Lamb[5] posed a scheme for grouping the monilias by fermentation and precipitin reactions. Peck and Hauser[6] analyzed the lipids of *C. albicans*. Kluyver and Custers[7] found that three strains of *C. albicans* were able to oxidize sucrose readily at slightly less than the rate for glucose. In their experiments the cells were obtained from growths on malt agar plates, washed, and suspended in phosphate buffer at pH 4.5. Van Niel and Cohen,[8] using a single strain of *C. albicans*, found that suspensions prepared directly from yeast-agar plates showed a high endogenous rate of oxygen consumption that declined rather rapidly with time. The addition of certain sugars to such suspensions brought about little increase in oxygen uptake. However, after starving the cells for several hours by aeration in water or buffer, the suspension did oxidize added substrate. The quantity of oxygen theoretically necessary for complete oxidation of the added substances was rarely consumed. The oxidation process ceased with only one-third of the glucose or sucrose oxidized, the remainder of the sugar being presumably converted to an assimilation product of the general formula CH_2O. Similarly, pyruvate, acetate, and ethanol were incompletely oxidized, the percentage of substrate oxidized calculated on the basis of oxygen consumption being 60, 50, and 63%, respectively. Using carbon dioxide release as a criterion of completeness of reac-

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tion, the figures obtained checked quite well with these.

Bernheim and Gale[9] showed that β-propiolactone was inhibitory to the growth of certain pathogenic fungi. This compound had previously been reported by Hartman et al[10] to be inhibitory to certain viruses and bacteria, and its toxicity had been shown to be rather low in mice[11–13] Gale[14] studied its effects on the metabolism of *Blastomyces dermatitidis*.

The present work was undertaken to determine the effects of β-propiolactone* (Betaprone) on the growth and metabolism of *C. albicans* in vitro and to attempt to indicate possible mechanisms of its action.

MATERIALS AND METHODS

The organism used was obtained from Dr. N. F. Conant of the Medical Mycology Department at Duke Hospital. It was grown for 24 hours on Kelley's agar slants without blood.[15] Cells were harvested by taking them up in water, centrifuging 3 minutes at 2000 r.p.m. resuspending in buffer (*M*/20 Na-K phosphate, pH 6.0 or 7.4) and centrifuging for 3 minutes. This latter process was repeated once, and the packed cells were resuspended in buffer in a final suspension of 1:20. In some cases the washing with buffer was omitted, and in some cases washing was omitted altogether. In these latter instances the cells were simply taken up in a small quantity of buffer and then made up to 1:20 after centrifuging.

Manometric measurements were done in the customary Warburg apparatus at 37 C. The drug was usually added to the main compartment of the vessel, along with 1.0 ml of the cell suspension. Substrates were added from the side-arm in all but a few experiments. In measurements of oxygen consumption the center well contained 20% KOH; when measuring carbon dioxide release, 0.2 ml of 5.0% H_2SO_4 was added from the center well. The vessels had a fluid volume of 2.2 ml and an atmosphere of air.

Ammonia was determined according to the method already described.[16] Chromatographic analyses of amino acids were done according to Berry et al,[16] using Whatman no. 1 filter paper, a *n*-butanol-acetic acid-water solvent and ninhydrin indicator. "Free" amino acids within the cell were released by boiling the cells in 0.2 *N* HCl in a water bath for 10 minutes. This suspension was then neutralized with an equal portion of 0.2 *N* NaOH and chromatographed. The salt in this low concentration did not cause streaking.

Attempts to prepare cell-free enzyme extracts were done by rapid freezing and thawing, using a dry ice-acetone mixture, ultrasonic vibration (Raytheon apparatus, 9 kc), grinding in a Teflon homogenizer (Arthur Thomas & Co.) with Johns Manville Filter-Cel or Hyflo Super-Cel[17] and the preparation of an acetone-dried powder.[18] Only the last two procedures yielded preparations with satisfactory activity. Grinding did not give complete rupture, as shown by culture of the suspensions; however, a large amount of cell debris was evident upon microscopic examination, indicating at least partial disintegration of the organisms.

Controls were run in all cases with vessels which contained all components except the sub-

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* Several samples were generously supplied by the B. F. Goodrich Chemical Company.

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strate (or drug) being tested in that particular experiment; the substrate (or drug) solution was replaced with distilled water. All experiments were run in duplicate or triplicate.

RESULTS

Growth.—Turbidimetric measurements were done by following the growth of *C. albicans* in an Evelyn colorimeter with a 690 λ filter. In a nonprotein broth culture tube inoculated with 0.1 ml of a heavy aqueous suspension of cells and incubated at 37 C, Betaprone, in a concentration of 0.15 mg/ml, was bacteriostatic for 4 to 7 hours. At the end of this time, the drug was perhaps oxidized, and growth proceeded, indicated by a rise in turbidity. In a concentration of 0.50 mg/ml, growth was completely inhibited up to 76 hours. Control tubes showed a nearly logarithmic increase in turbidity.

Endogenous metabolism.—Betaprone in concentrations up to 0.25 mg/ml was itself oxidized by *C. albicans*. If the drug were added at 37 C, the end points obtained were 3 moles oxygen and 3 moles carbon dioxide/mole. These are the quantities required for compete oxidation of the drug to carbon dioxide and water. If, however, the drug were added at room temperature, immediately brought to 37 C, and measurements begun, the rate of oxidation was much higher and no definite end points could be obtained. The RQ of the oxidation under the former conditions was 0.98; under the latter it was 0.90. Making corrections for the different amounts of gases exchanged under the two sets of conditions at the end of a certain period, the RQ for the increased gaseous exchange when the drug was added at room temperature compared with the exchange when added at 37 C was 0.95, indicating that the increased metabolism was at the expense of endogenous carbohydrate. Varying the period of preincubation at room temperature did not alter the pattern significantly; the increase was approximately the same with periods from 1 to 30 minutes. No explanation is readily apparent for this phenomenon.

This type of stimulation was not evident in ground cells. In this case, there was actually an inhibition of the endogenous metabolism which was proportional to the concentration of Betaprone used.

Ammonia was released in the oxidation of Betaprone, but there was no significant difference in the amounts released under the two sets of conditions mentioned above. At the end of 4 hours there were 4.4 γ ammonia/ml in the vessels in which Betaprone had been added at 37 C. When the drug was added at room temperature and brought to 37 C, the concentration of ammonia was 4.6 γ/ml.

ATP was without effect on the oxidation of the drug in intact cells.

Glucose.—In contrast to the findings of Van Niel and Cohen,[8] this strain oxidized glucose at a rapid initial rate with an oxygen uptake of 2 moles/mole glucose utilized. Betaprone depressed the rate somewhat, but not the final end point of the reaction (fig. 1a). Essentially the same behavior was noted at pH 6.0 as at pH 7.4. Grinding the cells had little effect on the oxidation, but did increase the degree of inhibition (fig. 1b). Thus it would seem that the grinding process, which exposed the cellular contents to the external medium, brought about a contact of the drug with the enzymes necessary for the oxidation of glucose. This indicates that in intact cells, the drug does not reach these enzymes in as great concentration, if at all, implicating the cell surface as the site of action.

Ammonium sulfate, in a concentration of 0.2 mg/ml, did not increase the initial rate of glucose oxidation, but did raise the end point to approximately

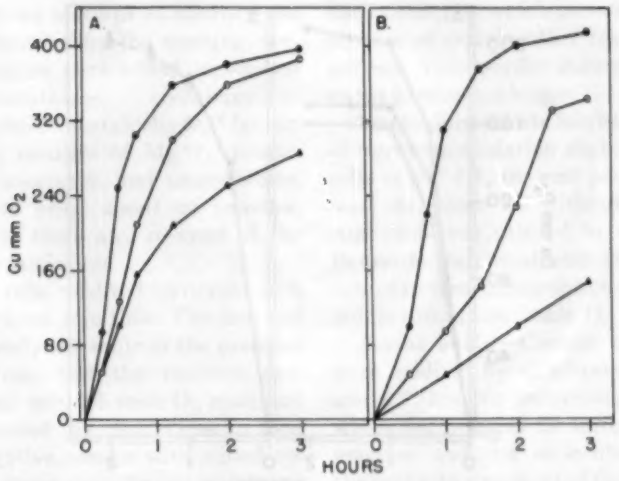

FIG. 1.—The oxidation of glucose by (A) intact and (B) ground cells of *C. albicans* at pH 6.0 and the effect of Betaprone thereon. ●——●, cells+glucose (5.0 μM/ml); ○——○, cells+glucose +Betaprone (0.125 mg/ml); ▲——▲, cells+glucose+Betaprone (0.25 mg/ml). The proper controls have been subtracted.

2.5 moles O_2/mole. Ammonia was utilized in this process. This assimilation was depressed by Betaprone in a concentration of 0.125 mg/ml (table 1).

Intermediate products of carbohydrate metabolism.—Acetate was oxidized at a rapid initial rate, the end point being equivalent to 1 mole O_2/mole (fig. 2a).

TABLE 1.—*The assimilation of ammonia by* C. albicans *during resting respiration and during the oxidation of pyruvate and glucose, and the effect of Betaprone on these processes.*

Flask contents	NH_3 added, μg/ml	NH_3 recovered, μg/ml	Percent NH_3 utilized
Cells+pyruvate	0.0	0.0	
Cells+pyruvate+NH_3	54.5	38.2	30
Cells+glucose	0.0	0.0	
Cells+glucose+NH_3	54.5	35.5	35
Cells only	0.0	0.0	
Cells+NH_3	54.5	48.5	11
Cells+pyruvate+Betaprone	0.0	3.2	
Cells+pyruvate+Betaprone+NH_3	54.5	49.2	10
Cells+glucose+Betaprone	0.0	4.1	
Cells+glucose+Betaprone+NH_3	54.5	47.8	12
Cells+Betaprone	0.0	4.6	
Cells+Betaprone+NH_3	54.5	56.9	0

Concentration of glucose and pyruvate, 2.5 μM/ml; of Betaprone, 0.125 mg/ml. The pH was 7.4, and the duration of the incubation was 4 hours. Ammonia was added as ammonium sulfate.

The rate was slowed slightly by Betaprone, and the end point was lowered (0.7 moles/mole). This depression did not occur in ground cells (fig. 2b). This again could be indicative of the cell surface being the primary site of action of the drug.

Pyruvate, at pH 7.4, was oxidized at an initially slow rate which increased with time. The end point was 1 mole O_2/mole. This reaction was markedly inhibited by Betaprone (fig. 3a). In order to attempt to explain this lag period, various procedures were applied. First, it was attempted to "adapt" the cells to pyruvate. However, resting cells preincubated with 30 γ/ml pyruvate for 1 hour before the addition of the substrate quantity displayed no increased initial rate when the latter was added at pH 7.4. Cells grown in a medium to which had been added 2.0% pyruvate showed only a slight increase. The rate was still not of the order of that of either glucose or acetate. Omitting the washing with buffer resulted in increased

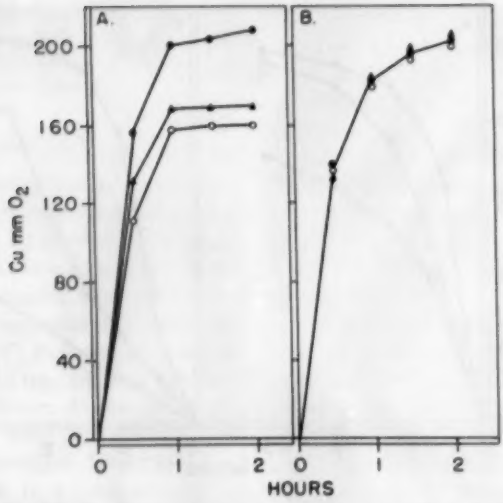

FIG. 2.—The oxidation of acetate by (A) intact and (B) ground cells of *C. albicans* at pH 6.0 and the effect of Betaprone thereon. ●——●, cells+acetate (5.0 μM/ml); ○——○, cells+acetate +Betaprone (0.125 mg/ml); ▲——▲, cells+acetate+Betaprone (0.25 mg/ml). The proper controls have been subtracted.

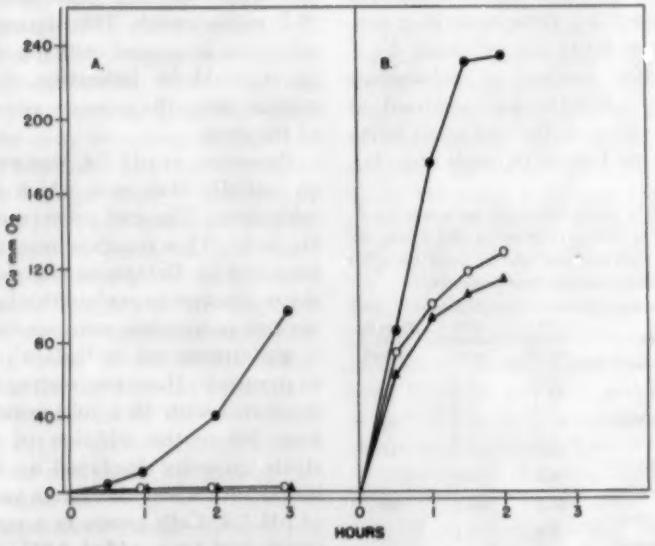

FIG. 3.—The oxidation of pyruvate by (A) intact and (B) ground cells of *C. albicans* at pH 7.4 and the effect of Betaprone thereon. ●——●, cells+acetate (5.0 μM/ml); ○——○, cells+acetate +Betaprone (0.125 mg/ml); ▲——▲, cells+acetate+Betaprone (0.25 mg/ml). The proper controls have been subtracted.

activity. In an attempt to identify the factor(s) removed by the washing, certain substances were added to washed cells. Glutathione, cocarboxylase, Mg^{++}, acetate,[19] acetaldehyde,[19] fumarate, and a mixture of Mg^{++}, malate, fumarate, succinate, and oxaloacetate, all failed to bring about an increase. Neither was there any reversal of the Betaprone inhibition.

Ground cells oxidized pyruvate at a linear rate from zero time. The rate was approximately the same in the presence of Betaprone, but the reaction proceeded until only 0.5 mole O_2/mole had been consumed (fig. 3b). Thus, in view of the negative results with added cofactors, it seems that the cell membrane is the limiting factor in pyruvate oxidation, and also determines the inhibition by Betaprone, up to a certain point. Beyond this, the drug seems to inhibit the further metabolism of some intermediate of pyruvate. This intermediate product is most likely not acetate in view of the amounts of oxygen consumed (1 mole/mole with each compound) and also the fact that Betaprone had almost no effect on acetate oxidation in ground cells.

An acetone-dried powder of the cells decarboxylated pyruvate (1 mole/mole) with no concomitant oxygen uptake, and this process was totally unaffected by Betaprone. Again, however, in view of the fact that the oxygen consumption was the same with both pyruvate and acetate, it seems improbable that the reaction pyruvate $\xrightarrow{-CO_2}$ acetaldehyde $\xrightarrow{(O)}$ acetate occurs in intact or ground cells. The observed carbon dioide release in this preparation may have been a reaction which occurs only in the absence of certain other (oxidative) reactions. This powder showed no endogenous gaseous exchange.

Ammonium sulfate increased the rate of pyruvate oxidation slightly in intact cells at pH 7.4; the end point obtained was the same as without ammonia. Ammonia was utilized in the process. Betaprone inhibited both the increased rate of oxygen consumption and the ammonia utilization (table 1).

Amino acids.—Certain amino acids were oxidized by *C. albicans* at pH 7.4 and 6.0, and an interesting correlation was noted between the initial rate of the reaction and the molecular weight of the acid with a number of the compounds tested (fig. 4). Other acids, however, did not fall into this pattern (glycine, leucine, valine, and aspartic acid), and this, combined with the fact that ground cells oxidized amino acids at a markedly depressed rate, if at all, argues against a simple, passive diffusion process. Chromatographic analysis showed disappearance from the supernatant fluid of the amino acids which were oxidized at the highest rates; nesslerization showed the simultaneous appearance of ammonia. The rates of these processes were proportional to the rates of oxygen uptake. Betaprone, in a concentration of 0.25 mg/ml, inhibited the oxygen uptake, disappearance of amino acids, and appearance of ammonia (fig. 5, table 2).

Incubated control cells, with and without drug, showed three ninhydrin-positive spots upon lysis. Incubation of cells with substrate plus drug gave no alteration of this finding. The added amino acids, in the presence of Betaprone, were recovered quantitatively from the supernatant fluid; none was taken into the cell.

Fatty acids.—Saturated *n*-fatty acid salts up to C_{18} were oxidized at a rapid

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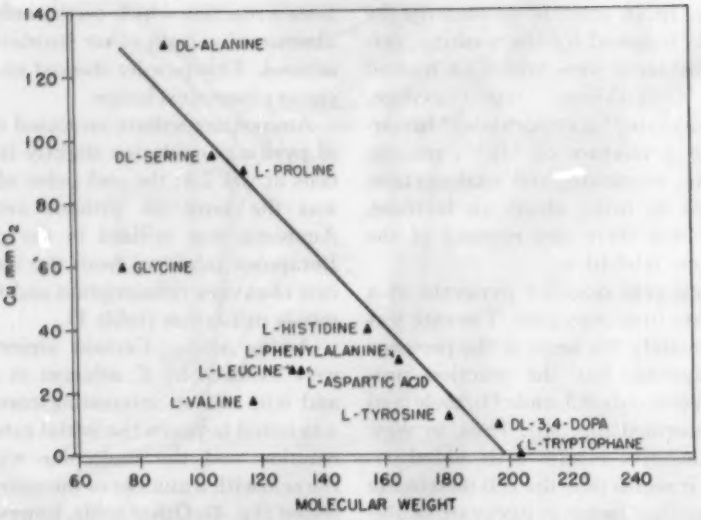

FIG. 4.—The initial rates of oxidation of certain amino acids by washed cells of *C. albicans* at pH 7.4 compared with the molecular weights of these compounds. The oxygen uptake values are for the first 30 minutes of each reaction minus the endogenous respiration for that same period. Concentration of substrates, 5.0 μM/ml.

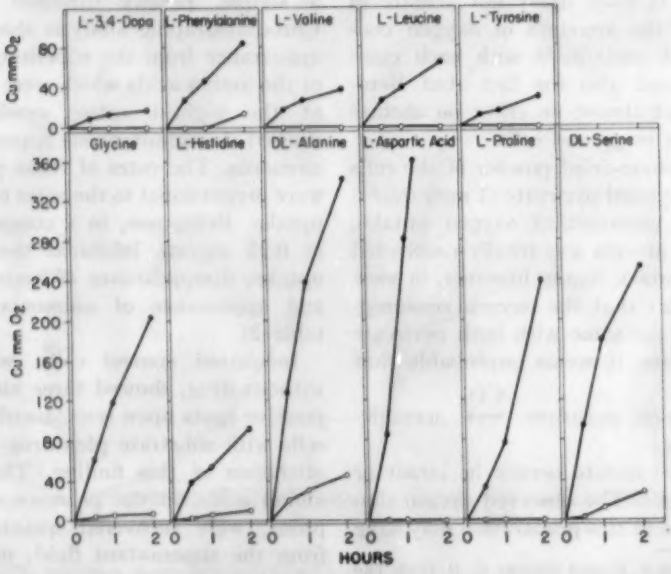

FIG. 5.—The effects of Betaprone (0.25 mg/ml) on the oxidation of certain amino acids by *C. albicans* at pH 7.4. Concentration of substrates, 5.0 μM/ml. The proper controls have been subtracted. ●——●, cells+substrate; ○——○, cells+substrate+drug.

TABLE 2.—*The deamination of certain amino acids by* C. albicans *and the inhibition of these processes by Betaprone.*

Substrate	NH_3 recovered (μM/ml)	
	With Betaprone	Without Betaprone
Dl-alanine	0.12	0.53
Dl-serine	0.11	0.88
L-phenylalanine	0.00	0.00
L-Tryprophane	0.00	0.00
L-Leucine	0.00	0.00
L-Valine	0.00	0.00
glycine	0.21	0.70
L-proline	0.06	0.56

Concentration of amino acids, 5.0 μM/ml; of Betaprone, 0.25 mg/ml. The pH was 7.4, and the duration of the incubation was 1 hour.

rate with definite end points (not shown on graph), and there was some depression by Betaprone (fig. 6). This inhibition was not as marked as occurred with certain other substrates, such as pyruvate or amino acids. Propionate, which is sometimes used as a therapeutic in vaginal moniliasis, was oxidized with an oxygen uptake of 2 moles/mole. The end points obtained with the other acids were not exactly proportional to the lengths of the carbon chains.

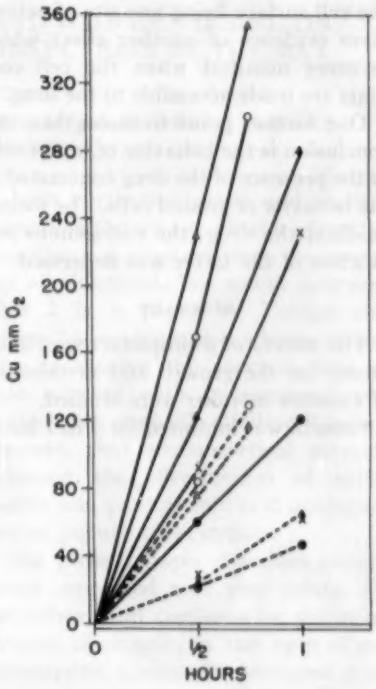

FIG. 6.—The oxidation of the sodium salts of certain fatty acids by washed cells of *C. albicans* at pH 7.4. Solid lines, cells+substrate (2.5 μM/ml); dotted lines, cells+substrate+Betaprone (0.25 mg/ml); closed circles, propionate; crosses, caproate; closed triangles, stearate; open circles caprylate; open triangles, palmitate. The proper controls have been subtracted.

DISCUSSION

The majority of evidence points to the conclusion that the cell surface or membrane is the primary site of action of Betaprone agaist *C. albicans*. In the case of glucose, which was oxidized at approximately the same rate in intact and in ground cells, the drug exerted its least effect. Such a similarity in behavior is best explained on the basis that the intact cell membrane is unnecessary for its metabolism. Thus, an agent which exerts its adverse influence on or near this membrane would be expected to be without significant effect on such a process.

Although intact cells were capable of oxidizing certain amino acids, this was depressed or completely inhibited in ground cells. Betaprone had a very marked effect on amino acid metabolism (oxygen uptake and deamination) in intact cells. Here is an example of a process or series of similar processes which apparently require an intact cell surface for maximal activity; these processes were depressed by the drug.

The metabolism of pyruvate in intact cells, which proceeded at an initially slow rate, was completely inhibited by a certain concentration of Betaprone. In ground cells, the rate was linear from zero time, and no inhibition by the drug was evident until one-half the amount of oxygen had been consumed as was taken up by intact cells. This phenommenon, while supporting a theory of

the cell surface being one site of action, gives evidence of another effect which becomes manifest when the cell contents are made accessible to the drug.

One further point to strengthen this conclusion is the behavior of intact cells in the presence of the drug contrasted to the behavior of ground cells. The former oxidized the drug; the endogenous respiration of the latter was depressed.

SUMMARY

The effects of β-propiolactone (Betaprone) on the growth and metabolism of *Candida albicans* were studied.

Growth was inhibited for 4 to 7 hours with 0.15 mg/ml, and for >76 hours with 0.50 mg/ml. The oxidation of added substrates such as glucose, amino acids, pyruvate, acetate, and fatty acids was inhibited. The oxidation of those substrates which apparently required an intact cell surface or membrane for their utilization was inhibited to the greatest extent.

The conclusion was drawn that the primary site of action of Betaprone is at or near the cell surface. A secondary effect which showed up in pyruvate metabolism was probably exerted at some area within the cell exposed by grinding.

THE EFFECTS OF CORTISONE AND OXOPHENARSINE ON *TRYPANOSOMA EQUIPERDUM* INFECTIONS IN THE RAT

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Cortisone enhances the severity of many experimental infections, including *Mycobacterium tuberculosis*[1] in rats, *Treponema pallidum*[2] in rabbits, poliomyelitis[3] in mice, *Diplococcus pneumoniae*[4] in rats, *Plasmodium relictum*[5] in pigeons, *Trypanosoma cruzi*[6,7] in mice, *Trypanosoma vickersi*[8] in monkeys, *Blastomyces dermatitidis*[9] in mice, and others.[10]

Robinson[11] has reported that in pneumococcal infections in rabbits penicillin was able to control the infection in both cortisone-treated and control animals although a greater dose of penicillin was required when coritosone was administered. We would interpret table 2 in a paper by Tobian and Strauss[12] as evidence that adrenocortical extracts shorten the survival time of mice infected with pneumococci and treated with penicillin. Vollmer[13] found, however, that adrenocortical extracts increased the effectiveness of sulfadiazine and pantothenic acid analogues against pneumococci in mice.

The present paper describes experiments concerned with two points: (1) the influence of cortisone on the effectiveness of arsenic, in the form of oxophenarsine, against *Trypanosoma equiperdum* in the rat, and (2) the course of *T. equiperdum* infections in the rat treated with cortisone.

MATERIALS AND METHODS

The rats were infected by intravenous injection of defibrinated, infected blood. In each experiment all the rats were anesthetized with ether and placed on individual animal boards for the injection. The femoral vein was exposed by a single snip of the scissors and the injection made with a tuberculin syringe equipped with a 27 gauge needle. By having all the rats ready at once in was possible to infect 12 or 14 rats in about 10 minutes. The cortisone was given subcutaneously. In the first experiment it was cortisone acetate Merck; in the second, cortisone acetate Upjohn, 25 mg per cc. Trypanosome counts were made with a hemocytometer at a dilution of 1:100 using 0.9% NaCl as the diluent. The trypanosomes in a volume equivalent to 0.001 cu mm of blood were counted in most instances. The charts in figures A to F are scaled to correspond with

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the numbers actually counted. At infection levels over 400,000 per cu mm or under 10,000 a smaller, or larger volume was examined as seemed appropriate.

RESULTS

In the first experiment (fig. A to G) 14 rats of 150 to 200 g were infected by intravenous injection of 0.4 ml of defibrinated infected blood. On the second day after infection two parasite counts, 5 to 6 hours apart, were made on each rat. These counts are found in table 1. Six of the rats were treated with cortisone according to the following schedule: On the 5th, 3rd and 1st day before infection and on the day of infection, 25 mg per kg; on the 1st day after infection, 75 mg per kg: on the 2nd day, 100 mg per kg; and on the 3rd, 4th, 5th and 6th days, 50 mg per kg. The first 3 doses were given once a day and the rest in 2 equal doses each day.

Figures A to G show the course of each infection for 5 hours before and 19 hours after injection of oxophenarsine. Each figure represents the infections in 2 rats, one treated with cotisone and the other not treated with cortisone, but both treated with oxophenarsine. The rats were paired according to similarity in weight before the first trypanosome count. Pairs A, C and E were males; pair D were females; pairs B and F were mixed, the cortisone treated rat being female in pair B and male in pair F. The untreated controls shown in figure G were females.

The 4 rats represented in figures A and B received oxophenarsine at a dose of 1 micromole per kg (0.258 mg per kg) intravenously after the second trypanosome count at the point indicated by the heavy arrows. Each infection continued upward during the next 3 hours, but in both figures the infection in the rat not treated with cortisone declined thereafter, while the infection in the rats given cortisone continued upward during another 3-hour period before beginning to decline. Nineteen hours after injection of oxophenarsine, in both instances, there was only about ½ an many trypanosomes in the rats not given cortisone. Figure G shows the course of the infections of figure A for a period of 3 days. After 24 hours the infection in the cortisone-treated rat began to increase and death occurred at about the 48th hour of observation. The infection in the rat not given corti-

TABLE 1.—*Number of trypanosomes per 0.001 cu mm of blood in rats treated with cortisone and in untreated controls.*

	Cortisone				Control			
	First count		Second count		First count		Second count	
	Actual	Calculated for 200 g rat	Actual	Calculated for 200 g rat	Actual	Calculated for 200 g rat	Actual	Calculated for 200 g rat
				Experiment 1				
	152	148	285	276	127	127	309	309
	167	137	293	240	197	154	363	312
	165	170	336	346	159	180	312	354
	159	181	355	405	167	167	335	335
	208	174	339	283	187	200	323	345
	245	233	402	382	193	164	325	276
Mean	183	174	335	322	172	165	328	322
				Experiment 2				
	210	208*			231	244*		
	226	182			254	185		
	269	223	460	382*	206	198	449	431*
	197	174	465	410	267	252	482	426
	206	180	449	392	186	146	379	305
	231	202	442	388	222	184	618	512
Mean	223	195	454	393	231	201	482	418

Table 2 contains the time data for these counts.
* Count calculated for 300 g rat in all instances in experiment 2.

TABLE 2.—*Data on* T. equiperdum *infections in 24 albino rats, 12 of which received cortisone.*

First count		Second count		Time between counts in hours	Generation time in hours	Time from beginning of infection in hours	
Actual	Calculated at 42.4 hours	Actual	Calculated at 47.9 hours			1st count	2nd count
Trypanosomes per cu mm in thousands							
			Experiment 1				
			Cortisone-treated				
152	180	285	360	5.0	5.5	40.8	45.8
167	186	293	330	5.5	5.9	41.4	46.9
165	176	336	343	5.9	5.7	41.8	47.7
159	162	355	322	6.0	5.1	42.3	48.8
208	183	339	303	5.3	7.6	43.6	48.9
245	208	402	340	5.2	7.7	44.2	49.4
Mean 183	182	335	333		6.4	42.4	47.9
			Controls				
127	145	309	375	5.0	3.9	41.2	46.2
197	216	363	410	5.6	6.3	41.6	47.2
159	163	312	319	6.0	6.2	42.1	48.1
167	163	335	309	5.9	5.9	42.7	48.6
187	162	323	280	5.5	7.0	43.7	49.2
193	160	325	271	5.1	6.9	44.4	49.5
Mean 172	168	328	327		6.0		
			Experiment 2				
			Cortisone-treated				
210	228†	*				46.2	
226	242	*				46.3	
269	279	460	495†	5.6	7.3	46.6	52.4
197	195	465	470	5.7	4.6	47.1	52.9
206	196	449	432	5.6	5.0	47.3	53.4
231	210	442	429	5.6	6.0	47.7	53.3
Mean 223	225	454	456		5.7	46.9	53.0
			Controls				
231	252	*				46.2	
254	275	455	520	5.3	6.3	46.3	51.9
206	209	449	470	5.6	5.0	46.8	52.7
267	258	482	488	5.6	6.3	47.2	52.9
186	167	379	360	5.6	5.4	47.8	53.4
222	197	618	572	5.5	3.8	47.8	53.4
Mean 231	226	477	482		5.4		

* No second count was obtained before exophenarsine injection in these 3 instances.
† Counts calculated at 46.9 and 53 hours in 2 experiment.

sone continued at a low level until the 72nd hour of observation. The infection later reappeared and killed this rat on the 9th day of observation.

In the rats represented by figure B there was not so striking a difference in the subsequent course of the infections. The cortisone-treated rat was found dead on the morning of the 6th day and the other rat died during the morning.

The 4 rats represented by figures C and D received oxophenarsine at a dose of 2 micromoles per kg given intravenously. In all instances the infection declined promptly and rapidly. Considering figures C and D together, one would not suspect any antagonism between arsenic and cortisone from these 2 experiments although one of them does show a suggestion of it.

The 4 rats represented by figures E and F received oxophenarsine at a dose of 4 micromoles per kg. The infection declined very rapidly and similarly whether cortisone had been given or not.

To summarize these results, cortisone interfered with a low dose of oxophenarsine but not with a higher dose, when the therapeutic effect is judged by the reduction of the trypanosome count during the hours immediately following the administration of oxophenarsine. However, when the subsequent course of the infection was considered, an interesting relationship was observed. This is seen in table 3, where the survival times of the rats are recorded. Considering all the rats that received 2 micromoles of arsenic or more, it will be observed that in every case the cortisone-treated rat relapsed and died by the 14th day, while the rats not receiving cortisone survived at least until the 18th day and in 2 instances were still

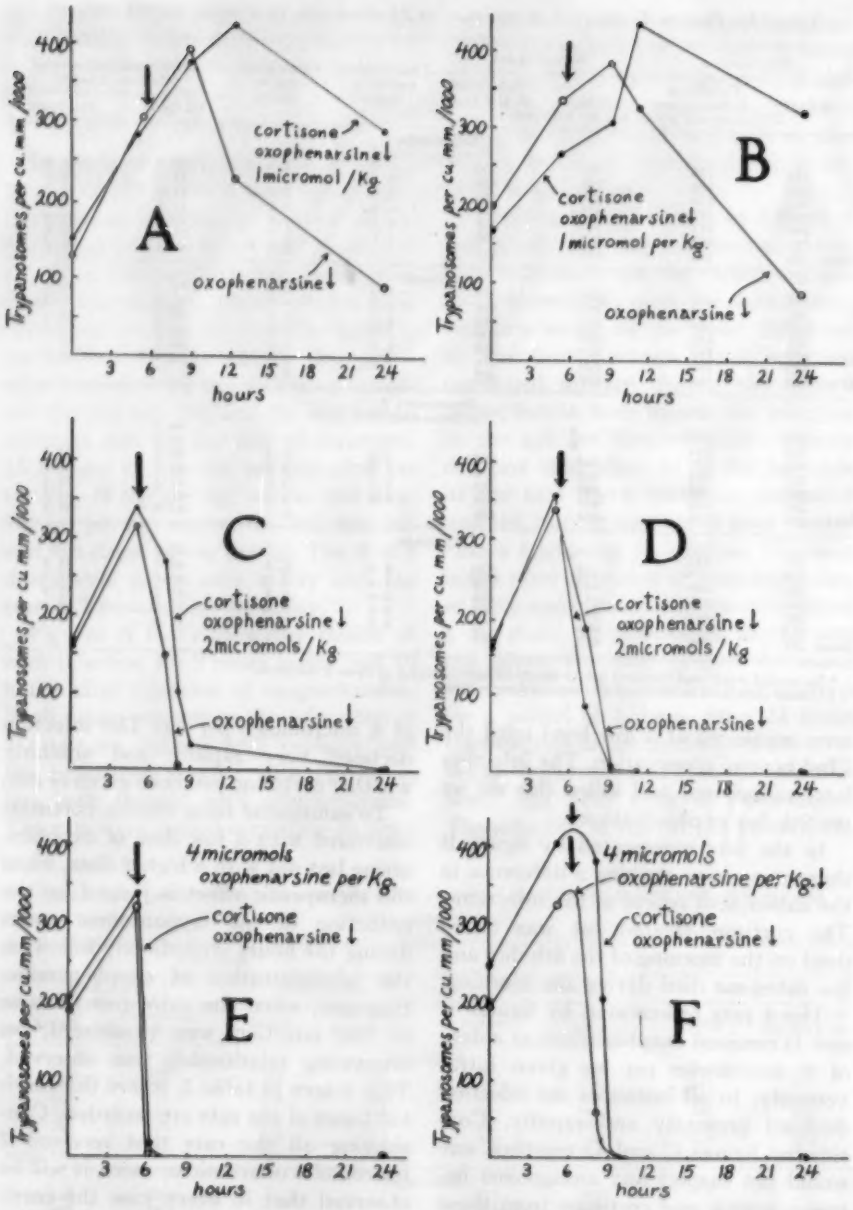

FIG. A and B.—Course of infection in 4 rats treated with 1 micromole of oxophenarsine per kg, 2 of which also received cortisone.

FIG. C and D.—Infections in 4 rats treated with 2 micromoles of oxophenarsine per kg, 2 of which also received cortisone.

FIG. E and F.—Infections in 4 rats treated with 4 micromoles of oxophenarsine per kg, 2 of which also received cortisone.

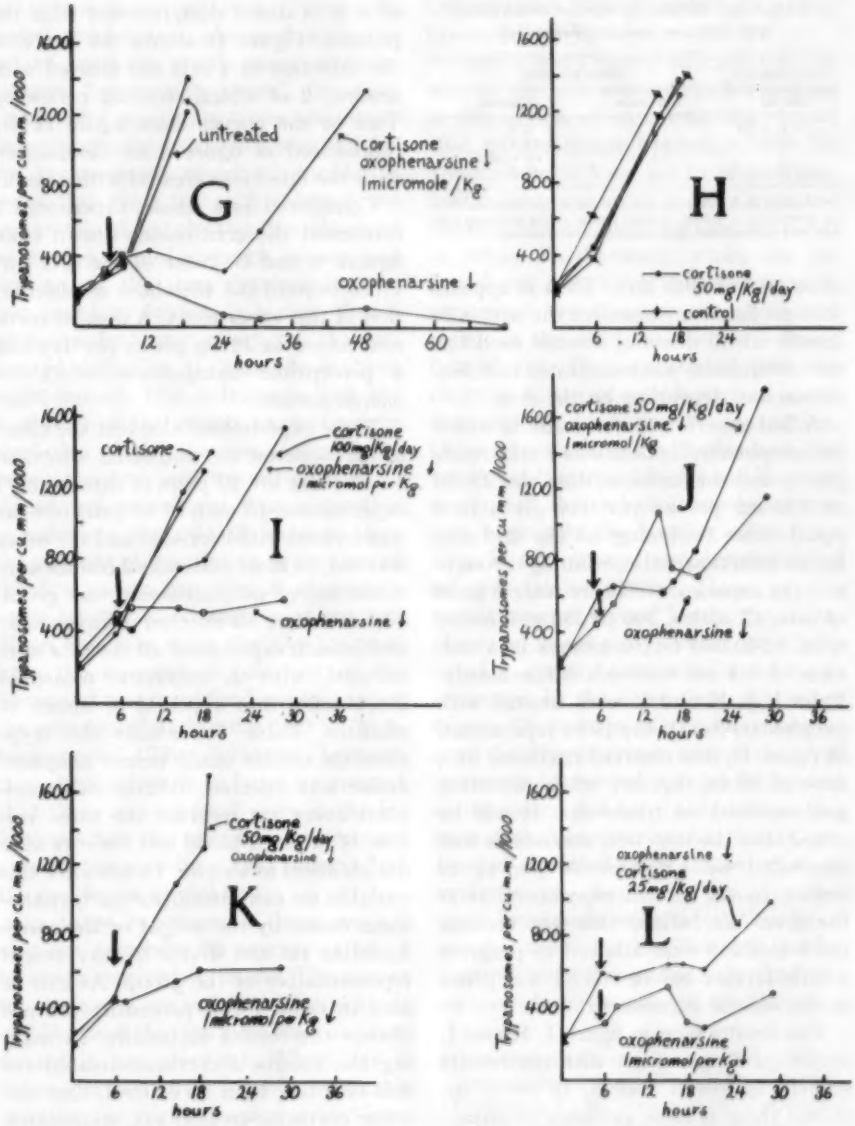

FIG. G.—Infections in two untreated controls shown with the two infections of figure A for a period of 72 hours.

FIG. H.—Infections in 4 rats, 2 of which received cortisone and 2 no treatment.

FIG. I.—Infections in 2 rats treated with 1 micromole of oxophenarsine per kg, one of which received cortisone at a dose of 100 mg per kg per day, and 2 rats not treated with oxophenarsine, one of which received cortisone at a dose of 50 mg per kg per day.

FIG. J and K.—Infections in 4 rats treated with 1 micromole per kg of oxophenarsine, 2 of which received cortisone at a dose of 50 mg per kg per day.

FIG. L.—Infections in 2 rats treated with 1 micromole of oxophenarsine per kg, one of which received cortisone at a dose of 25 mg per kg per day.

TABLE 3.—*Survival of infected rats treated with cortisone and oxophenarsine.*

Oxophenarsine dose micromoles per kg	Days survived	
	Cortisone	Control
1	5, 5	5, 9
2	11, *	18, 37
4	12, 14	60+, 60+
0		4½, 5

* One rat died on the 5th day but not of the infection. This rat gave birth to a litter of dead young on the previous day, was very anemic and died despite a transfusion.

alive on the 60th day. Thus it appears that cortisone antagonizes the action of arsenic at all doses of arsenic used but the antagonism was manifested in different modes, depending on the dose.

A 2nd experiment was made in which the oxophenarsine dose was 1 micromole per kg and the cortisone dose was 25, 50 or 100 mg per kg per day given in 2 equal doses beginning on the 2nd day before infection and continuing throughout the experiment. There were 6 pairs of rats, all males, 200 to 250 g, infected with 3,540,000 trypanosomes in a volume of 0.4 ml injected intravenously. Pairs I, J, K and L were treated with oxophenarsine. Of the pairs represented in figure H, one received cortisone at a dose of 50 mg/kg/day while the other pair received no treatment. It will be noted that the infection was not as well controlled by 1 micromole per kg of arsenic in the second experiment as in the first. We believe this was because the infections were allowed to progress a little farther before arsenic was given in the second experiment.

The results seen in figure I, K and L are in good agreement with the results seen in figures A and B. In every instance there is clear evidence of antagonism between cortisone and arsenic. Figure J, if considered alone, could not be used as evidence in favor of any particular theory about the relationship of these substances. Considered with the other curves, part of figure J fits into the pattern of the others while a part of it is in direct disagreement with the pattern. Figure H shows the curve of the infection in 4 rats not treated with arsenic, 2 of which received cortisone. Two of the curves from figure H are reproduced in figure I for comparison with the infections treated with arsenic.

Considered as a whole, experiment 2 confirmed the conclusions drawn from figures A and B. None of the rats survived beyond the 48th hour of observation in this experiment. A dose of cortisone as low as 25 mg per kg per day had a perceptible antagonistic effect for oxophenarsine.

These experiments also test the effect of cortisone on the untreated infection. In all there are 13 pairs of rats in the 2 experiments. In each of 12 pairs one rat was treated with cortisone and the other was not. In most instances 2 counts were made before oxophenarsine was given. The rats were all infected intravenously and in each experiment all the rats were infected with a carefully measured inoculum within a period of about 10 minutes. Table 1 contains the trypanosome counts made before oxophenarsine was injected. Within an experiment every rat received the same volume of infected blood but the rats were not identical in weight. To allow for this variable we can multiply each trypanosome count by the weight of the corresponding rat and divide by any weight representative of the group. As can be seen in table 1 this procedure did not change the results materially. In making the counts a cortisone-treated rat was counted, then its control, then another cortisone-treated rat, its control, etc. to avoid systematic differences due to changes during the counting period, which lasted around 3½ hours. How well this scheme succeeded is evident in table 2, where we have set down the actual counts as well as the same counts adjusted to the mean time of the counts

on cortisone-treated rats. These adjusted values were obtained with the aid of semi-log graph paper and the average "generation times" for each group. The generation times are meant to represent only the time required for the trypanosome count to double. A generation time for each infected rat was obtained by plotting the observed counts on semi-log graph paper and reading off the time required for the first count to double. It is clear that if cortisone had any effect on the untreated infection it must have been slight indeed. This is borne out by the similarity of the counts as well as the similarity of the changes observed between the first and second counts. In the second experiment it is interesting to calculate the rate of increase from the moment of inoculation to the time of the first count, assuming a reasonable value for the blood volume of the rat. When this was done the average rate from the moment of infection to the first count was considerably higher than the rate found during the next 5½- to 6-hour period. The difference between these two values is so large we suspect that there was a period early in the infection when the parasitemia increased at a higher rate than that observed at the end of the second day.

In these experiments there were 6 rats not treated with arsenic at all, 2 of which received cortisone. The curves for these rats are in figures G and H. It will be noted that the time scale on these figures is not identical. When this is taken into account it is evident that this infection is highly reproducible from time to time and that cortisone was without effect on the course of infection.

DISCUSSION

These results were considered in relation to the experiments of Pfeiffer and Tatum,[14] Bunting, Pfeiffer and Tatum,[15] Dubois and Kohn[16] and Reiner and Leonard.[17]

Pfeiffer and Tatum,[14] in a study of the role of the reticulo-endothelial system in the action of oxophenarsine, found that trypanosomes treated in vivo for 5 minutes with 5 mg per kg of oxophenarsine disappear more rapidly from the blood in the rat than from drawn blood in vitro. Furthermore, when the infected rat was killed 5 minutes after treatment the trypanosomes survived longer in the dead rat than in a living treated rat. They concluded that the curative action of oxophenarsine is not due entirely to a direct drug action. Other experiments by Pfeiffer and Tatum[14] and Bunting, Pfeiffer and Tatum[15] implicated the reticulo-endothelial system, especially the Kupffer cells of the liver, in the removal and destruction of treated trypanosomes. These experiments were of several kinds: (1) transplants of organs of treated rats indicated a greater accumulation of trypanosomes in the liver than any other organ, (2) ligation of the liver lengthened the disappearance time of the trypanosomes in treated rats while ligation of the spleen did not, (3) in histopathological studies they found that in untreated infected rats there was no evidence of phagocytic activity but upon treatment with 5 mg per kg of oxophenarsine the Kupffer cells quickly became actively phagocytic and destroyed large numbers of the drug

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treated trypanosomes within 24 hours.

Dubois and Kohn[16] have questioned the importance of phagocytes in the action of arsenic on the basis of their experiments in which treated trypanosomes disappeared just as rapidly in splenectomized and blockaded rats as in intact controls. However, Pfeiffer and Tatum believed that the liver was the major site of destruction of treated trypanosomes.

The adverse effect of cortisone on the phagocytic system of the rat has been studied recently by Heller,[18] using radioactive, colloidal chromium phosphate. He reported a 50% inhibition of phagocytosis caused by cortisone at a dose of 20 mg per rat for 3 days. Besides its effect on the filtering action of the phagocytes, cotisone also delays the destruction of ingested material in the macrophages. Kass and Kendrick[19] found that erythrocytes injected subcutaneously in cortisone-treated rats persisted within the macrophages of the regional lymph nodes for several days after disappearing from controls. Lurie, Abramson, Heppleston and Dannenberg[20] have reported persistence and even proliferation of bacteria in macrophages of cortisone-treated animals after their disappearance in control animals.

The experiments of Reiner and Leonard[17] were concerned with the importance of treated trypanosomes as antigen, and with the action of antibody in conjunction with drug action. They found that the serum of treated rats had agglutinating and lytic effects not shown by normal rat serum. Reiner and Chao[21] reported the immunization of rats to *T. equiperdum* by the use of vaccines. Immunized rats survived longer than controls but eventually died of the infection. Mayer and Brousseau[22] have observed the immunizing effect of treating mice, infected with *T. equiperdum*, with neoarsphenamine or melaminylphenyl stibonic acid. Browning and Gulbransen[23] reported this phenomenon earlier with *Trypanosoma brucei* and arsacetin.*

The inhibitory action of cortisone on antibody production has been established by the observations of Bjoernboe, Fischel and Stoerk,[24] Germuth, Oyama and Ottinger,[25] Malkiel and Hargis,[26] Eisen and coworkers,[27] Germuth and coworkers,[28] and Fischel, Vaughan and Photopoulos.[29]

In the present experiments cortisone given at a dose of 25 to 125 mg per kg per day beginning 1 week before infection was demonstrably antagonistic to

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* The immunizing effect of treating trypanosome infections with drugs was known to Ehrlich and Shiga[30] in 1904 and was intensively studied by Terry[31,32] before 1911.

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the early therapeutic effect of a low dose (1 micromole per kg) of oxophenarsine but not antagonistic to the early therapeutic effect of higher doses (2 or 4 micromoles per kg). In other words, the blood is cleared of trypanosomes by oxophenarsine just as rapidly in a cortisone-treated rat as in a rat not given cortisone, provided oxophenarsine is given at a dose of 2 micromoles per kg or more. However, a late effect of cortisone was found, inasmuch as the cortisone-treated rats relapsed and died sooner than the rats treated with oxophenarsine alone.

In the light of the various studies we interpret the present results in the following way. When a low dose of arsenic (1 micromole per kg) was given to an infected rat, many of the trypanosomes were weakened but not killed in the blood. In this state the trypanosomes fell prey to the macrophages, which were unable to capture the untreated fully active trypanosomes. In those rats given the cortisone phagocytic function was impaired and the effectiveness of a low dose of arsenic diminished. When a higher dose of arsenic was given many of the trypanosomes were killed in the blood and the phagocytic function became relatively less important in the early action of the drug. The subsequent course of the infection depended on the ability of the rat to form antibody in response to the ingested trypanosomes. In the cortisone-treated rats antibody production was impaired and as a result the infection soon relapsed and killed the rats.

These results are interpreted as evidence that the rat is an active participant in the early action of a low dose of oxophenarsine but not in the early action of a higher dose of oxophenarsine. On the other hand, the rat is capable of actively resisting the parasite once the drug has cleared the blood of most of the parasites. Since cortisone has no effect on the normal course of the untreated infection nor on the ability of higher doses of oxophenarsine to clear the blood this late effect of cortisone would seem to depend on its ability to interfere with acquired immunity.

In view of the ability of cortisone to interfere with natural and acquired immune mechanisms these experiments are evidence that the untreated rat infected with *T. equiperdum* represents an instance of parasitism uncomplicated by resistance on the part of the host. This might be suspected from the high degree of virulence, the uniformity of the infection and the exponential course of this invariably fatal infection. The departure of the course of the parasitemia from an exponential curve late in the infection suggests a destruction of a portion of the population but we have found, in another type of experiment,[33] that this departure is probably due, at least in part, to an inhibition of growth. The present experiments strengthen the argument that the departure from the exponential curve is not due to any mechanism of acquired immunity, at least none that can be inhibited by cortisone.

SUMMARY

Cortisone interfered with the action of oxophenarsine on *Trypanosoma equiperdum* in two ways, depending on the dose of oxophenarsine. At a low dose of oxophenarsine cortisone caused a delay in the disappearance of trypanosomes from the blood. At higher doses cortisone administration was associated with earlier relapse and a higher death rate than treatment with oxophenarsine alone.

Cortisone had no effect on the course of *T. equiperdum* infections in the absence of oxophenarsine.

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COMPETITIVE VALUES OF SMOOTH AND MUCOID *BRUCELLA ABORTUS* IN MIXED INFECTIONS IN MICE

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A factor which suppressed the establishment of nonsmooth types in liquid cultures of smooth brucellas was observed by Braun (1949) in normal serums of species of animals relatively susceptible to infection with brucellas. Serums of relatively resistant species failed to suppress the establishment of variants.

Braun and Hauge (1948) have presented data which appeared to establish a correlation between the absence of the smooth selective (SS) factor from the serums of various animal species and the ability of nonsmooth variants to establish themselves in the animal tissues. In these experiments mice, which lack the SS factor, were inoculated with massive doses of variant brucellas and sacrificed after 5 weeks. At this time variants identical with the particular type inoculated were isolated from the spleens of almost all mice. Guinea pigs inoculated with large numbers of nonsmooth cells did not yield variants when cultures were made 9 to 10 weeks after inoculation.

In further studies of the significance of the SS factor Braun (1950) found that during the course of infection with smooth (S) organisms the selective factor disappeared from the serums of the infected animals which normally have it, e.g., guinea pigs, cattle, human beings. Results reported by Braun and Mead (1949) indicated that one variant was able to establish itself in vivo during the period when the SS factor was not demonstrable. Eighteen days after infection of guinea pigs with a population of *Brucella suis* consisting of more than 99% S and less than 1% intermediate (I) types, the tissues of the animals yielded 18% of the I type upon culture. Similar cultures made 36 days after inoculation contained 81% I types.

Jones and Berman (1951) and Braun et al (1951) have shown that when guinea pigs were inoculated with S brucellas and subsequently inoculated with mucoid (M) variants, at a time when the SS factor had disappeared, the M variants were unable to maintain themselves in the host tissues. In fact, the variants were eliminated more rapidly from such guinea pigs than from those inoculated simultaneously with the S and M cells. In the experiments performed by Braun's group, the M organisms were inoculated by either the subcutaneous or the intracardial route. The variants persisted slightly longer when inoculated intracardially but were still eliminated more rapidly than were variants inoculated simultaneously with S. These observations indicated that in eliminating the SS factor from the serums of the guinea pigs by infection, a specific immunity was developed, thus complicating the evaluation of the significance of the SS factor and of factors inherent in the organisms which may condition the ability of variants to establish themselves in animals. One experimental approach to the problem of evaluation of the competitive values of variant and S organisms in vivo is offered by the use of animals, such as mice, which lack the SS factor.

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MATERIALS AND METHODS

Brucella abortus strain 2308, which was obtained originally from the Bureau of Animal Industry, U. S. Department of Agriculture, and a streptomycin resistant (Sr) mucoid variant (Sr 2308 M) derived from it were used exclusively in this investigation. The parent strain is sensitive to streptomycin (Ss) and its colonial morphology is smooth. These cultures were used in the experiments with guinea pigs reported by Jones and Berman (1951). The use of a mucoid strain which was resistant to streptomycin facilitated differentiation of the organisms recovered from animal tissues.

Inoculums were prepared by suspending 24 to 48 hour growth from Albimi agar slants in physiological saline. These were adjusted to a concentration of approximately 10^9 cells per ml, as determined by turbidity measurements with a Coleman Nephocolorimeter. Appropriate dilutions of these suspensions were inoculated into mice or onto cell count plates from which the numbers of cells in inoculums were calculated.

CFW mice from the colony maintained by this department were used in all experiments. At the time of inoculation the mice weighed 20 to 25 g. When animals were sacrificed, macerated tissue from spleens, livers, testes, and at least two pair of lymph nodes was cultured on 2–1 agar (Henry, 1933) and on 2–1 agar containing 200 to 500 μg of streptomycin per ml. After incubation for 4 days at 37 C the plates were examined with a binocular dissecting microscope using obliquely transmitted light, and the colonial morphology of the recovered organisms was recorded. These observations were supplemented by the acriflavine test and the crystal violet test of White and Wilson (1951).

EXPERIMENTAL RESULTS

Experiment 1.—Mice were inoculated intraperitoneally with numbers of strain Sr 2308 M ranging from 10^6 to 10^9, with numbers of the parent Ss 2308 S ranging from 10^3 to 10^6, and all possible combinations. Three mice from each group inoculated with S or M organisms alone and two from each group inoculated with the combinations were sacrificed at 4, 14, 28 and 56 days after inoculation.

The results are summarized graphically in figures 1 and 2. It can be seen from figure 1 that the mucoid organisms persisted in large numbers for at least 28 days in the tissues of mice inoculated only with the mucoid culture. At 56 days after inoculation the tissues yielded relatively few mucoid organisms. There was no evidence of "reversion" to the smooth type in any of the mice.

Examination of the sites of localization of the mucoid organisms in these mice indicates a possible preferential localization in the spleen as compared with the lymph nodes. This was particularly apparent in mice inoculated with 10^6 or 10^7 organisms and sacrificed at 14 and 28 days.

An inoculum of 10^3 of the smooth organisms was sufficient to establish an infection which persisted for 56 days in most mice. Clear-cut differences in site of localization were not apparent in these mice. The only gross pathologic changes observed were moderate lymphadenitis and splenomegaly.

In animals inoculated with mixtures of M and S (fig. 2) and sacrificed after 4 days, the mucoid organisms predominated. In fact, smooth organisms could be demonstrated in the tissues of only 8 of 32 mice and in most of these relatively few were present. No organisms could be isolated from 5 mice. Excessive contamination may explain the failure to isolate brucellas from at least two of them. Cultures of the lymph nodes of one mouse inoculated with 10^6 M and 10^6 S yielded 25% M colonies and 75% S, while the spleen contained 70% M and 30% S.

At 14 days, mixtures of M and S were recovered from all but 8 of 32 animals and 4 of these had been inoculated with the largest numbers of mucoid organisms (10^9). Mucoid organisms predominated in cultures from most animals, especially those inoculated with only 10^3 or 10^4 S organisms.

There was strong evidence of preferential localization by the two types in

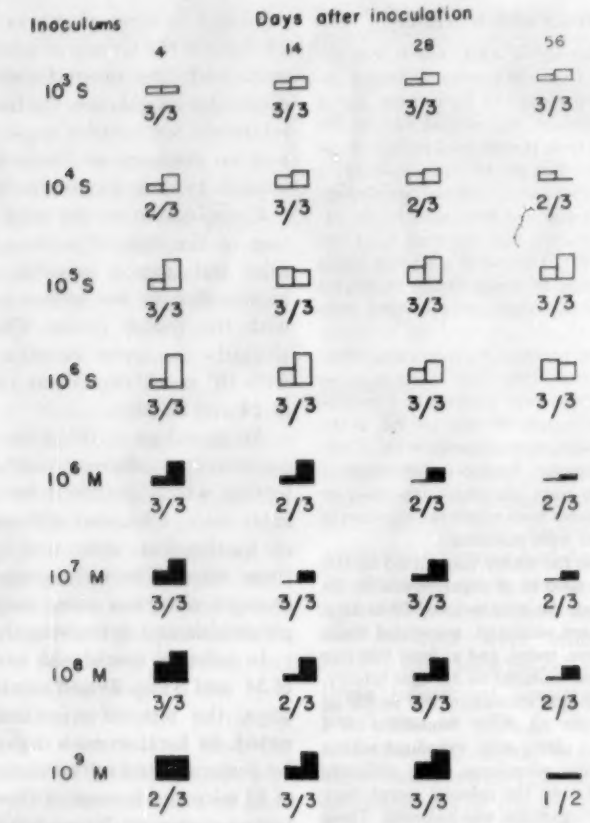

FIG. 1.—Relative numbers and colonial types of organisms recovered from groups of mice inoculate with varying numbers of S or M *Br. abortus* (exper. 1).

Left bar—lymph nodes, right bar—spleens, open bar—smooth, solid bar—mucoid. Fractions are number of infected animals over number examined.

some mice. For example, one mouse inoculated with 10^4 S and 10^6 M had only smooth organisms in the lymph nodes and only mucoid organisms in the spleen. The lymph nodes of a mouse inoculated with 10^4 S and 10^7 M yielded 99% S colonies and 1% M, while the spleen contained 99% M and 1% S. Various degrees of this type of distribution were apparent in animals examined at this time.

At 28 days, mixtures of M and S were recovered from the tissues of 19 mice, M alone from 8, S alone from 2 and no recoveries were made from 3. Four of the mice which yielded M alone had been inoculated with 10^9 M and 10^3 or 10^4 S, the other four had been inoculated with smaller numbers of M and 10^3 or 10^4 S. The tendency toward differential localization of the two types in spleen and lymph node was still apparent in many animals. It was also evident from the proportions of the two types that the smooth organisms were establishing themselves at the expense of the mucoid organisms.

At 56 days after inoculation, mix-

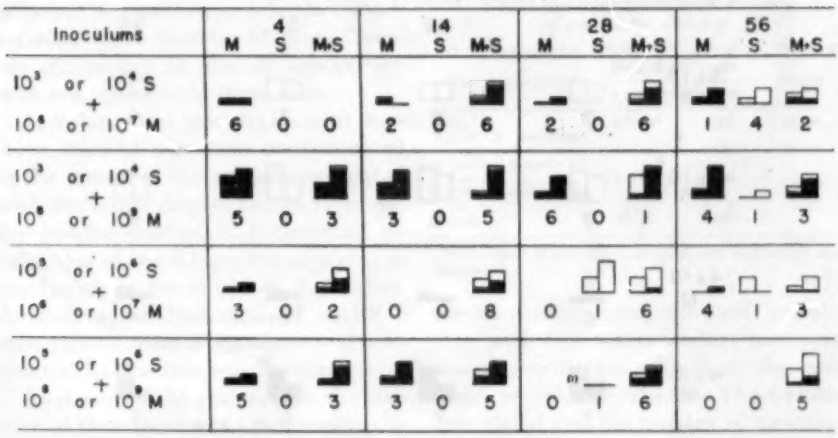

FIG. 2.—Relative numbers and colonial types of organisms recovered from groups of mice inoculated with mixtures of S and M *Br. abortus* (exper. 1).

Left bar—lymph nodes, right bar—spleens, open bar—smooth, solid bar—mucoid. Eight mice were examined in each group except the one inoculated with 10^5 or 10^6 S and 10^8 and 10^9 M sacrificed at 56 days. Six mice were examined in this group. Numerals below bars give numbers of mice whose tissues yielded all M, all S or mixtures of M and S organisms. Mice from which brucellas were not isolated are not included.

(1) Two S colonies were recovered from this animal.

tures of the two types were recovered from the tissues of 13 mice, M alone from 9, S alone from 6 and no isolations could be made from 2. Differential localization was still manifest in some animals, and there was an increased proportion of smooth organisms in the animals from which mixtures were recovered, as well as the increase in number of animals infected with smooth organisms alone.

Experiment 2.—To confirm and extend the findings presented above, the experiment was repeated using fewer combinations of numbers of organisms and larger groups of mice. Groups of mice were inoculated intraperitoneally with 5×10^3 or 5×10^6 cells of Ss 2308 S, or 4×10^6 or 4×10^8 cells of Sr 2308 M, respectively. Three groups of mice received combinations of 5×10^3 S with 4×10^8 M, 5×10^6 S with 4×10^6 M, or 5×10^6 S with 4×10^8 M. Groups of 3 to 7 mice from each of the control lots, and 6 from each of the groups given mixtures were sacrificed at 9, 16, 31 and 58 days following inoculation.

The results are summarized in figures 3 and 4. It may be seen that the M organisms persisted in relatively large numbers throughout the 58 day observation period in the tissues of mice inoculated with 4×10^8 cells, and at least to some extent in the mice given 4×10^6 cells. "Reversion" from M to S was observed in 10 of the 26 mice examined which had been inoculated with the larger number of organisms, and from one of the 20 animals which had received the smaller dose. These smooth brucellae were indistinguishable from the parent S form of strain 2308 when streaked together with it on 2-1 agar, but were resistant to streptomycin, as was the M variant inoculated into the mice. The S form did not predominate

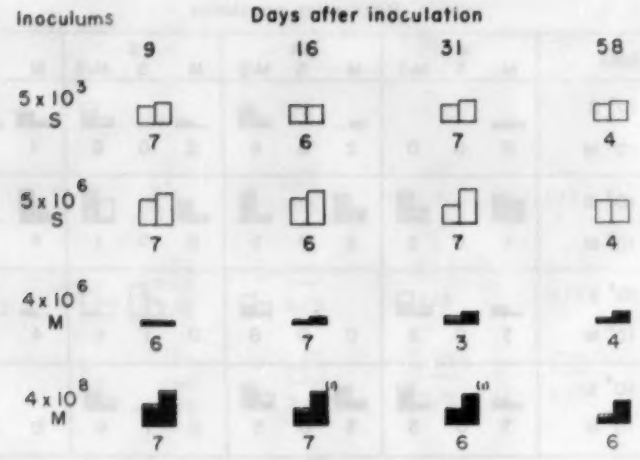

FIG. 3.—Relative numbers and colonial types of organisms recovered from groups of mice inoculated with varying numbers of S or M *Br. abortus* (exper. 2).

Left bar—lymph nodes, right bar—spleens, open bar—streptomycin sensitive smooth, cross-hatched bar—streptomycin resistant smooth, solid bar—mucoid. Numerals below bars give numbers of mice in each group. All mice examined were infected.

(1) A few S colonies were recovered from these tissues.

Days after inoculation

Inoculums	9 M	9 S	9 M+S	16 M	16 S	16 M+S	31 M	31 S	31 M+S	58 M	58 S	58 M+S
5 x 10^3 S + 4 x 10^8 M	2	0	4	0	0	6	0	0	6	0	1	4
5 x 10^6 S + 4 x 10^6 M	0	2	4	0	0	6 (1)	0	4	2	0	6	0
5 x 10^6 S + 4 x 10^8 M	0	1	5	0	0	6	0	1	5	0	0	5 (1)

FIG. 4.—Relative numbers and colonial types of organisms recovered from groups of mice inoculated with mixtures of S and M *Br. abortus* (exper. 2).

Left bar—lymph nodes, right bar—spleens, open bar—smooth, solid bar—mucoid. Numerals below bars give numbers of mice whose tissues yielded all M, all S or mixtures of M and S organisms. Proportions of smooth colonies which were resistant or sensitive to streptomycin were not recorded. All animals examined were infected.

(1) A few M colonies were recovered from these tissues.

in any of the animals in which reversion had occurred but was present in relatively greater numbers in the lymph nodes than in the spleens of the mice in which it was found. Relatively more M organisms were recovered from the spleens than from the lymph nodes of most of the mice.

An inoculum of 5×10^3 S cells was sufficient to establish an infection which persisted in all mice for 58 days. Clear-cut differences in site of localization were not apparent in these mice.

At 9 days after inoculation with mixtures, mucoid organisms predominated in the tissues of the animals inoculated with the larger dose of the M type and the smaller dose of S. Preferential localization of the S type was apparent in the lymph nodes of two of these mice. Animals which had received 5×10^6 S cells yielded more S organisms and preferential localization was manifest.

As shown in the graph, with the passage of time there was a progressive displacement of M brucellas by the S type in the tissues of the mice. This was most apparent in the animals which had received approximately equal numbers of the two types, and was merely delayed in the animals which had been inoculated with larger numbers of M organisms. Differential localization of S organisms in the lymph nodes and the M type in the spleens was observed in most of the groups.

Experiment 3.—Large inoculums of M organisms which produced no signs of toxicity when injected intraperitoneally into mice had a lethal effect when introduced intravenously (authors, unpublished data). In a titration experiment 8×10^8 M cells killed 2 of 13 mice 36 to 40 hours after intravenous injection; 8×10^9 M cells were lethal for 10 of 13 mice treated in the same way.

This experiment was designed to study the effect of prior intraperitoneal inoculation with S brucellas on this toxic effect and on the persistence of M organisms in any mice that might survive the intravenous inoculation. Groups of mice were injected intravenously with 9×10^8 or 9×10^9 M brucellas after one of the following treatments: intraperitoneal inoculation with S 8 weeks earlier, intraperitoneal inoculation with S 4 weeks earlier, intraperitoneal inoculation with S at the same time, no other treatment. The number inoculated and the number of survivors in each group are shown in table 1. The survival curves in figures 5 and 6 indicate that prior infection of mice with smooth *Br. abortus* increased their susceptibility to the toxic effect of large numbers of M cells introduced intravenously. Signs of toxicity were evident within a few hours in the groups which received the larger number of M organisms and which had received S organisms 4 or 8 weeks previously. Thirty-three of the 34 mice died in less than 24 hours. Thirty-one of 33 mice exposed to the same number of M, but which had no previous experience with S, expired during the period from 24 to 260 hours after injection of M organisms.

TABLE 1.—*Survival of mice following intravenous inoculation with large numbers of mucoid* Br. abortus.

Smooth inoculums	Mucoid inoculums		Totals
	9×10^8	9×10^9	
None	17/18*	1/18	18/36
9×10^8 S simultaneously with M	16/18	1/15	17/33
1.1×10^8 S 4 weeks before M	13/17	0/16	13/33
9×10^8 S 8 weeks before M	3/17	0/18	3/35
Totals	49/70	2/67	

* Numbers of mice which survived over numbers of mice inoculated.

Of the animals which received the smaller inoculum of M organisms, 14 of 17 infected 8 weeks previously with S died within 90 hours; 4 of 17 infected 4 weeks previously with S died within 50 hours; but only 3 of 36 not previously infected failed to survive.

The mice which survived intravenous inoculation of 9×10^8 M organisms were divided into three groups which were subjected to postmortem examination at 14, 25 and 52 days after injection. The results are summarized graphically

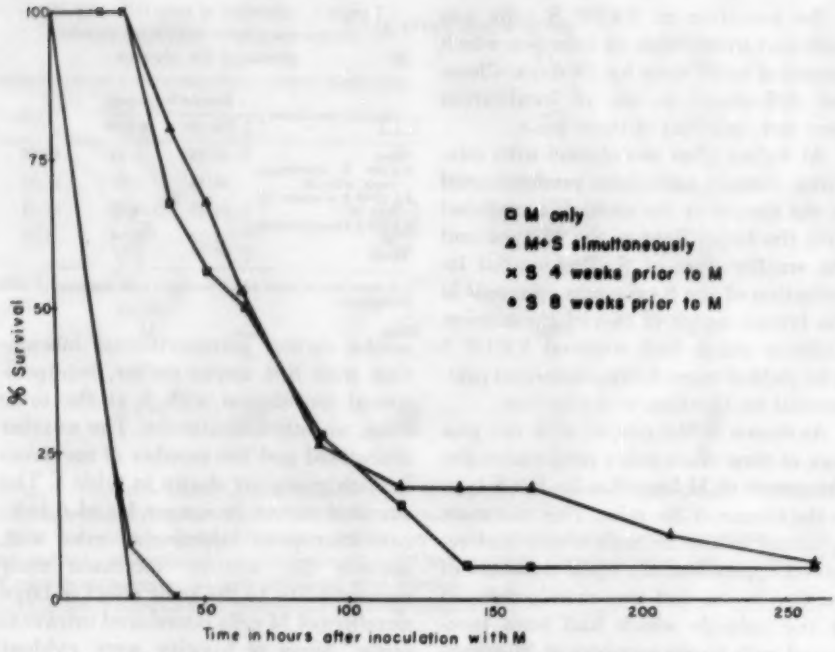

FIG. 5.—Survival curves of mice inoculated intravenously with 9×10^8 M *Br. abortus*.

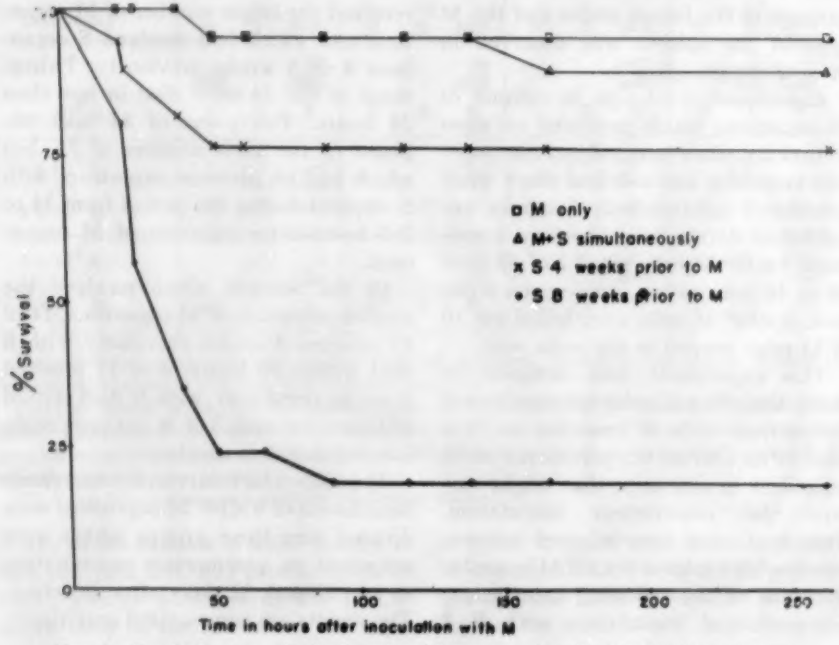

FIG. 6.—Survival curves of mice inoculated intravenously with 9×10^8 M *Br. abortus*.

in figure 7. The persistence and localization of the mucoid strain when injected intravenously did not differ significantly from that found after intraperitoneal injection in experiments 1 and 2. "Reversion" to S occurred in 6 of the 17 mice which received M alone. Prior infection with S brucellas had little, if any, effect on the persistence of M organisms in those mice autopsied 14 days after inoculation with M. There was a smaller proportion of M in tissues of the 4 mice which had received S 4 weeks before M (group 2) than in the 5 animals which received S and M simultaneously (group 1), but this difference had disappeared in animals sacrificed at 25 and 52 days. Mixtures were present in the majority of mice in groups 1, 2 and 3 and the superior competitive value of S organisms, at the expense of the M variant, was again evident with the passage of time. That "reversions" of M to S occurred was shown by the presence of Sr smooth organisms (as well as Ss) in tissues of 5 of the 32 mice which had been inoculated with both S and M organisms.

DISCUSSION

Comparison of the results of experiments 1 and 2 with those obtained by Jones and Berman (1951) from guinea pigs, inoculated simultaneously with the same M variant of *Br. abortus* and the parent S type reveals several interesting differences as well as a general similarity. Although the M type was able to maintain itself in competition with S organisms more successfully in the tissues of mice than in guinea pig tissues, the tendency of S brucellas to establish themselves, from relatively small inoculums, at the expense of large numbers of M brucellas, was manifest in both species. However, when the

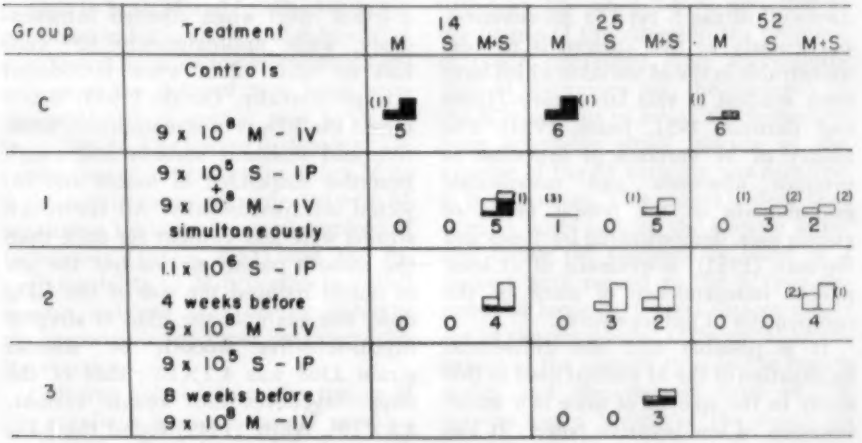

FIG. 7.—Relative numbers and colonial types of organisms recovered from groups of mice inoculated with *Br. abortus*—M, M and S simultaneously or M after previous infection with S (exper. 3).

Left bar—lymph nodes, right bar—spleens, open bar—streptomycin sensitive smooth, cross-hatched bar—streptomycin resistant smooth, solid bar—mucoid. Numerals below bars give numbers of mice whose tissues yielded M, S or mixtures of M and S organisms. All animals examined were infected.

(1) A few streptomycin resistant back mutants from M to S were recovered from these tissues. (2) A few M colonies were recovered from these tissues. (3) Six M colonies were recovered from this animal.

numbers of M variants were very large relative to the numbers of S organisms (as 10^8 M, 10^3 S) the variants persisted almost as long in animals inoculated with mixtures as in animals inoculated with the variants alone, although the numbers which could be recovered were smaller.

It is clear that in vivo selective conditions differ in animals which have or do not have the SS factor, and that the outcome of mixed infections in species which lack the SS factor depends in large part upon the numbers of variants inoculated. It is equally clear that even in hosts lacking the SS factor the competitive value of a variant with regard to the S type depends largely upon factors inherent in the organisms. This is most apparent when the numbers of the two types are more evenly in balance. These undefined factors might be considered to contribute to the "invasiveness" of the organism. It is interesting to speculate on the role of the "native antigen" of the S type in invasiveness, particularly as this antigen is not demonstrable in the M variants which have been studied in this laboratory (Jones and Berman, 1951, Jones, 1953). The ability of M variants of brucellae to produce abscesses and microscopic granulomata in the lymph nodes of guinea pigs, demonstrated by Jones and Berman (1951), is evidence of at least partial independence of some of the components of pathogenicity.

It is possible that the differential localization of the M variant used in this study in the spleens of mice is a manifestation of low invasive power. It has been pointed out that in animals inoculated intraperitoneally with M organisms alone, more organisms were recovered from the spleens, where apparently they are somewhat resistant to host defense mechanisms, than from the lymph nodes, where the defenses might be more effective. In mixed infections, this rapid clearance of M from the lymph nodes may tip the relative numerical balance in favor of the S forms, whose "invasive" factors seem to be more effective against the host defense. It may be postulated that the S organisms are delayed in establishing themselves in the spleens because of the numerical advantage of the M type, mediated by the lower efficiency of the local host defense. The ability of the S form eventually to displace the M type when the numerical balance is properly adjusted makes it unlikely that this delay is attributable to the accumulation in the spleen of metabolites inhibitory for the S type, produced by the M organisms (Jones and Berman, 1951).

Further evidence that the M variant used in this study is less pathogenic than S *Br. abortus* is found when these data are compared with the results obtained by other workers. In our experiments inoculums of 10^9 cells of Sr 2308 M had a lethal effect when injected intravenously, while approximately 10^{10} cells had no toxic effect when introduced intraperitoneally. Olitzki (1953) determined the LD_{50}'s of streptomycin sensitive and resistant smooth and rough brucellas suspended in mucin and injected intraperitoneally. All the rough strains were less virulent for mice than the smooth parent strains but the use of mucin reduced the size of the LD_{50} dose. For example the LD_{50} of streptomycin-sensitive smooth *Br. abortus* strain 2308 was 4.2×10^7; that of the streptomycin-resistant rough variant, 3.8×10^9. White (1949) found the LD_{50} of S cells of strain 2308 to be 4×10^9 when injected intraperitoneally without mucin into mice of the Rockefeller strain. One MLD of a recently isolated CO_2-sensitive strain of *Br. abortus* was 4×10^8 cells when inoculated into white mice by the intraperitoneal route with

or without mucin (Live and Giuliani, 1953). The apparent absence of the "native antigen" is a possible explanation for the failure of inoculums of M cells, 10 to 100 times the required number of S, to produce toxic death.

The survival curves in figures 5 and 6 indicate that infection with S brucellas produced a state of hypersensitivity which increased with time at least until eight weeks after inoculation. The reaction of the animals to introduction of large numbers of M cells was more rapid and more severe in those animals which had been exposed to S organisms. This is analogous with a type of anaphylactic hypersensitivity reaction elicited when 3×10^9 cells of a mucoid variant of strain 2308 were injected intraperitoneally into guinea pigs which had received S organisms several weeks previously (Redfearn, unpublished data). These manifestations of hypersensitivity are evidence that some of the same or similar antigenic components must be present in both M and S cells. Antigenic relationship between M and S brucellas manifested by cross hypersensitivity reactions of the delayed type have been demonstrated in guinea pigs by Redfearn (1953). The failure to demonstrate production of antibodies for either M or S antigens in guinea pigs infected with M organisms by any of several serological techniques (Jones, 1953) and the inagglutinability of M brucellas in anti-S serums indicate that deeper antigens are responsible for these hypersensitivity reactions.

Cultures isolated from the tissues of the survivors in experiment 3 revealed no striking effect of prior infection with S cells, or of route of inoculation of the M, on persistence of the variant organisms in mice. Mucoid cells were recovered in approximately equal numbers at 25 and 52 days from mice which were given M and S, simultaneously, and from those that had received S organisms 4 weeks prior to M. A higher proportion of M organisms was present in the 3 animals sacrificed at 25 days which had been infected with S 8 weeks before M, but the number of animals is too small to permit conclusions. Large numbers of smooth brucellas persisted in mice for at least $11\frac{1}{2}$ weeks. Experiment 3 confirmed the conclusion drawn from experiments 1 and 2 that S organisms can establish themselves at the expense of variants. Localization of M in the spleens was apparent in all groups from which large numbers of M organisms were recovered.

SUMMARY

Mice were inoculated intraperitoneally with graded numbers of a highly pathogenic smooth strain of *Brucella abortus* and its mucoid variant, each alone and in combinations. Identification was facilitated by using a labelled (streptomycin-resistant) mucoid strain. Although the mucoid type persisted in the tissues of the mouse in the presence of the S organisms more successfully than in the guinea pig, the tendency of the S organisms to establish themselves from relatively small inocula at the expense of the M variants was manifest in both species. This makes it clear that even in hosts lacking the smooth selective factor the competitive value of a variant with regard to the S type depends in large part upon factors inherent in the organisms. Differential localization of the smooth type in the lymph nodes and the mucoid type in the spleens was a feature of the mixed infections.

Intravenous inoculation of large numbers of mucoid organisms had a lethal effect which was enhanced if the mice had experienced prior infection with smooth brucellae. Neither the change in route of inoculation nor previous in-

fection with smooth organisms affected the competitive values of the two colonial types in mice.

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A STUDY OF MEDIUMS FOR THE GROWTH AND ISOLATION OF FILAMENTOUS MOUTH ORGANISMS

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The existence in the mouth of filamentous nonbranching organisms which fail to grow or grow with difficulty on laboratory culture mediums has been recognized for some time. The history of these forms dates back to some of the earlier studies reported by Buehlmann,[1] Robin,[2] Trevisan,[3] Miller,[4] and a few others,[5,6] although their descriptions were not too complete. The first good description of the cultivation of nonbranching filaments was that done by Meunier and Bertherand[7] in 1898, while one of the few informative studies on the oral types of this group is that of Bibby.[8] The excellent review by Rosebury[9] aptly covers the work in this field, as very little has been done with these organisms since that time. Much of the work on these forms has been devoted to attempts at isolation and maintenance but little progress has been made in spite of many efforts. One is still confronted with the situation of finding large numbers of these forms in smears of plaque material, but on attempted cultivation few of them grow or, if they do appear, they fail to grow after one or more transfers.

This work dealing with the study of filamentous mouth organisms has centered primarily on those nonbranching forms which can be seen in direct smears of plaque material taken from human teeth. They could be distinguished from "typical" *Actinomyces* by their characteristic growth on agar and by morphology in stained smears taken from isolated colonies. The colonies of these forms appear filamentous or rhizoid in nature, with a rather soft consistency, in contrast to the hard, brittle appearance of most *Actinomyces* colonies. They occur in varying sizes and shapes, which may indicate the existence of several species in this group. In the past these forms have had an uncertain status in classification and have been referred to variously as *Actinomyces*, *Leptotrichia*, *Leptothrix*, and other terms.

MATERIALS AND METHODS

Various mediums were used by the earlier workers. They included modifications of brain-heart infusion,[10] use of blood from different animals,[10–12] other enrichment additions such as

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ascitic fluid,[13,14] use of inhibitory substances[11] such as dyes to make the medium more selective, and others. The technique of alternately transfering[9] the culture from one medium to another has also been suggested. In the beginning of this work we tried several different mediums in an attempt to find one which could be used both as an isolating medium and a medium in which the cultures could be adequately maintained for further study. These included nutrient agar, veal infusion agar, brain-heart infusion agar, Loeffler's serum agar, thioglycollate agar, blood agar (sheep's blood), chocolate blood agar, tomato juice yeast extract agar, tryptose agar, a casein hydrolysate medium, and a few others. Along with these solid basal mediums variations of semisolid and liquid mediums were tried. Other changes in the mediums involved the addition of various combinations of reducing agents, compounds known to bind inhibiting substances, "undefined" nutrients, and ions which might induce filamentation. The reducing agents included thioglycollate, cysteine, and glutathione. The nutrient additions were yeast extract, crystalline bovine serum albumin, ascitic fluid, saliva, egg yolk suspended in a special salt solution, powdered enamel from extracted human teeth, and distilled water extracts from the original plates containing numerous filamentous colonies. Other things such as Tween 80 and ergosterol were also tried. The sources of inorganic ions were Mg salts, Ca salts and powdered enamel. The substances used to block any inhibiting properties present in agar or the medium were charcoal, starch and serum albumin.

After screening various combinations, a medium composed of tryptose, starch and yeast extract proved to be the most promising. The composition of this medium is presented in table 1. This medium and three others considered to be promising were tested in a comparative study of their ability to support growth of filamentous forms in primary isolations. The other three mediums were: brain-heart infusion agar (BH), brain-heart infusion thioglycollate crystalline bovine serum albumin agar (BHTS), and dextrose veal infusion yeast extract agar (DVYA). The choice of BH was based on the fact that this medium closely resembled those used by the earlier workers in the isolation of these forms. BHTS was an attempt at improving the BH medium by the addition of a reducing agent and a possible "detoxifying carrier" as suggested by some work with leptospira[15] and the Reiter

TABLE 1.—*Tryptose starch yeast extract agar (TSY).*

Tryptose (Difco)	20 g
NaCl	5 g
Yeast Extract (Difco)	5 g
Soluble Starch (Pfanstiehl)	20 g
Agar	15 g

Medium adjusted to pH 6.8 to 7.0 and made to 1000 ml with distilled H_2O.

treponeme.[16] DVYA was used because of its simplicity and also because it has been and is being used to maintain many stock cultures known to have a wide assortment of nutritional requirements. TSY was chosen as a possible source of peptides that would not be supplied by the other infusions. Soluble starch was used because of a recent report by Nickerson[17] in which filamentation was induced in *Candida albicans*, and also because of the possibility that it could remove inhibitory properties of agar as demonstrated by work with *Neisseria gonorrhoeae*.[18,19] Yeast extract was included to supply any unknown growth factors that might be necessary. All of the mediums tested throughout this work were adjusted to a pH of 6.8 to 7.0 with NaOH before autoclave sterilization. Heat labile substances, such as bovine serum albumin, ascitic fluid, etc., were sterilized by filtration through sintered glass and added aseptically to the medium after sterilization.

The inoculums for the primary isolations were obtained from tooth scrapings taken largely from between the cuspid and bicuspid teeth of the mandible. Occasionally material was obtained from between two molars or between the bicuspid and first molar. In some of the earlier experiments special precautions were taken to obtain the material as free from external contamination as possible. This was done by the use of a separator, careful washing of the area and the use of cotton rolls.[20] The interproximal area was washed in the usual manner and dried with air and the plaque was removed by means of a sterile, modified dental scaler. The plaque was then suspended in 1.0 ml of sterile buffered saline, broken up by agitation and streaked on various solid mediums. In later experiments sterile instruments were used without the aid of the separator or cotton rolls to

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obtain the necessary material. The mouth was thoroughly rinsed with water prior to obtaining the specimens with no other precautions taken to prevent contamination. The material so obtained was usually a bit of the plaque and materia alba which was emulsified in sterile buffered saline and streaked with the aid of a wire inoculating loop on the plating mediums. Generally the material obtained by such a method when smeared and stained showed these forms in great abundance.

The primary plates, which had been streaked with the material from the tooth scrapings, were incubated in vacuum jars which were evacuated and flushed with CO_2 twice and finally re-evacuated until approximately 5% CO_2 partial pressure remained. These jars were then placed in an incubator at 37 C for a period of 3 to 4 days. The system used for evacuating these jars was a "Cenco Hyvac" vacuum pump attached to a mercury manometer to measure the degree of vacuum produced in the entire system. With the primary plates no other precaution was necessary since the mixed bacterial flora used up the remaining O_2 and thus allowed the filamentous forms to grow. In subsequent transfers, however, pyrogallic acid and sodium carbonate was used in the bottom of the jars to measure the degree and efficiency of the anaerobiosis obtained and also to remove the O_2 that remained after the evacuation procedure.

RESULTS

Of the various mediums used only four appeared to be promising, and these were used in a comparative study of colony formation of the filamentous bacteria. The other mediums mentioned were abandoned after preliminary trials since they rarely supported growth of these forms or because the nature of the medium made it difficult to isolate or to see the colonies.

Comparative studies of the primary plates of the four mediums were made in ten different series to determine the numbers of filamentous forms appearing on each medium. These plates were made in triplicate in each series. Due to the method of inoculating the plates an exact numerical comparison could not be made although a relative comparison was justified. The uniformity with which the bacteria were dispersed in the suspension used to streak the plates depended upon how well the material was broken up; since it was obtained largely as a mat in the dental plaques the material had a tendency to stick together and thus it was difficult to obtain a uniform suspension. Consequently, one could not be sure that all plates received an equal amount of material. Also, since recognition of colonies was based on colonial morphology this could well be another limiting or error-producing factor. The filamentous forms might conceivably produce a different colonial morphology on the various mediums used; or, certain mediums might favor filamentation and thus increase the number of these forms appearing on a given plate. To eliminate some of these variables these experiments were carried out several times and the results compared.

Admittedly, there was a wide variation in the numbers appearing on the different plates of the same medium in the different experiments and the proportionate differences between the various mediums were not always the same; but generally speaking the patterns were similar. The results of these series of experiments are shown in table 2. Where the counts were low the whole plate was observed, while in those cases where the counts were high only the colonies on a part of the plate were counted. In these cases generally one-third or one-half of the plate was

TABLE 2.—*Number of filamentous colonies appearing on plates of different mediums.*

Experiment	DVVA	BHTS	BH	TSV*
1	5	10	10	60+
2	6	12	11	60+
3	4	20	5	60+
4	3	15	6	60+
5	4	12	5	50+
6	3	20	6	40+
7	7	26	15	60+
8	6	28	12	60+
9	6	20	18	60+
10	8	30	20	60+

* Only part of these plates were counted since there were so many colonies on each plate. These figures thus represent only an approximation of the total number of filamentous colonies appearing on each plate.

counted. The main interest was to make a qualitative comparison between the various mediums so that one could use the best medium for their primary isolation and further maintenance. From the results obtained TSY supported the growth of more filamentous colonies in the primary isolations than did the other three mediums tested.

The filamentous colonies grew out as several distinctly different morphological types. Some examples of their colonial morphology are shown in the figures. All of the colonies showed streaming thread-like projections extending from the edges which varied in thickness from single filaments to several together. After 3 or 4 days of incubation these colonies measured 0.25 mm to 2 or 3 mm in diameter, and because of the small size the counts and isolations had to be done with the aid of a dissecting microscope with 20× magnification. One of the difficulties in counting these colonies was that these forms would appear under colonies of other bacterial species, thus making an accurate count impossible.

The pictures of the microscopic morphology of these forms, taken from stained smears, were somewhat similar in every instance, except that some were gram-positive and others were gram-negative. They varied somewhat in length and thickness; in general, the thicker, heavier forms were gram-positive or gram-variable, while the thinner forms were gram-negative. There were others that appeared as gram-negative sheaths containing gram-positive granules of varying sizes scattered throughout the length of the sheath.

Although the four mediums tested were able to support the growth of these forms in primary isolation from tooth scraping suspensions, they failed in carrying them through successive transfers. Several attempts were made to isolate and purify these cultures and they were found nonviable after one or two transfers. That is, every attempt at transfering ended in failure to maintain the cultures.

Modifications were made of the original TSY medium in an attempt to find a medium which would support the growth of these forms through successive transfers. Among the many modifications used the addition of Ca^{++} ions in the form of $CaCl_2$ in relatively small amounts (0.01%) and dextrose in 0.1% concentration improved the viability of these forms and cultures were thus carried through to the eighth transfer, at which time they were lost. It was also discovered that, for some unknown reason, after the medium had stood in the refrigerator for 3 to 4 weeks it failed to support the growth of these forms, even in primary isolation. It was therefore necessary to use fresh medium within a week or two after being made. A liquid medium of similar composition, minus the agar, and with half the amount of soluble starch, also supported the growth of some of these cultures. The growth in broth appeared as a clumpy sediment at the bottom with some clumps sticking to the sides of the tubes, leaving the broth clear. These broth cultures seemed to die for no apparent reason along with those kept on the agar plates.

In some of the earlier work it was observed that certain samples of soluble starch did not give as good a result as others. Several attempts were made to find the cause for this discrepancy in the different samples. After washing a sample of Pfanstiehl's soluble starch three times with warm redistilled water (large volumes of around 500 ml for every 150 g of starch), centrifuging each time and decanting the supernatant to remove any traces of reducing sugars and other water-soluble components, it

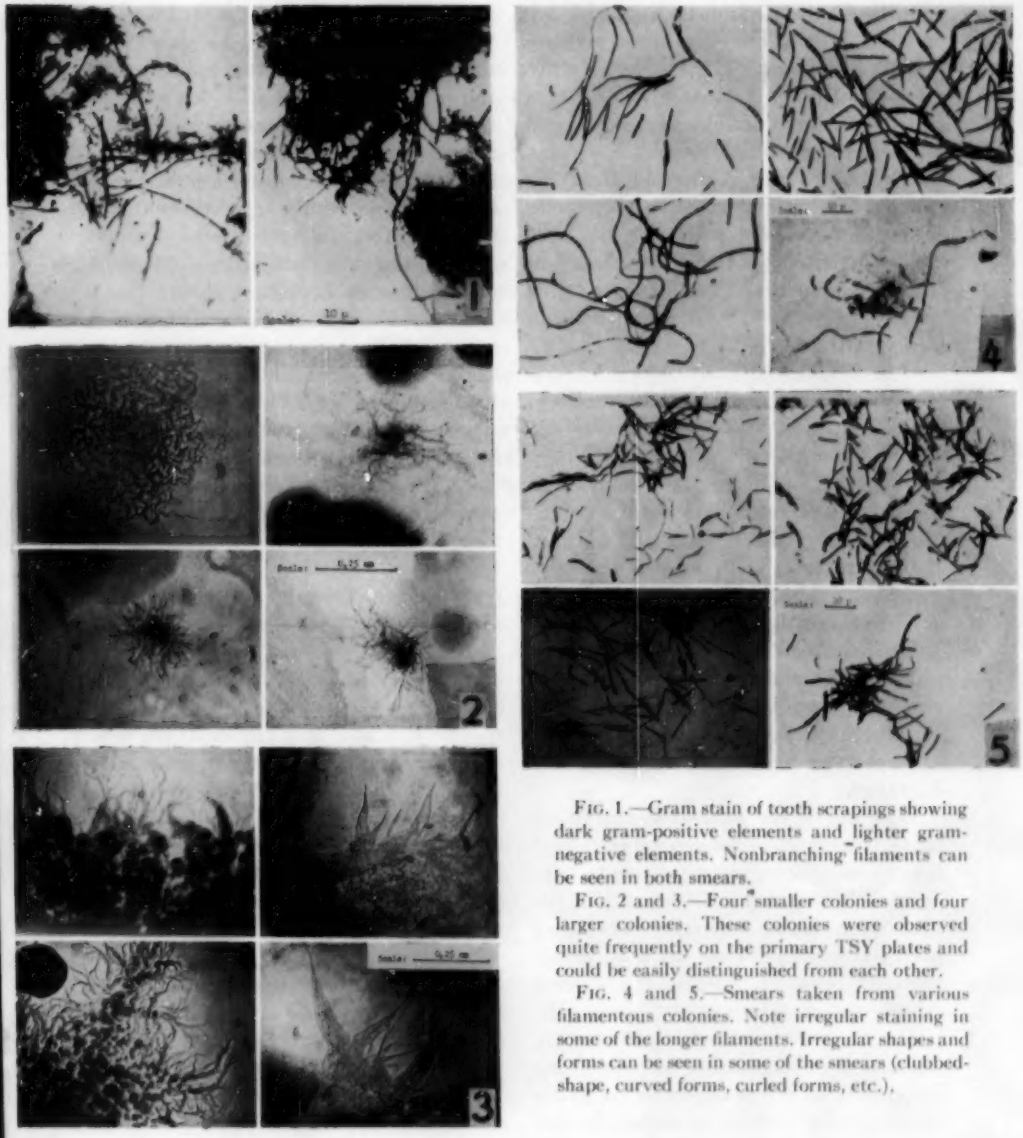

FIG. 1.—Gram stain of tooth scrapings showing dark gram-positive elements and lighter gram-negative elements. Nonbranching filaments can be seen in both smears.

FIG. 2 and 3.—Four smaller colonies and four larger colonies. These colonies were observed quite frequently on the primary TSY plates and could be easily distinguished from each other.

FIG. 4 and 5.—Smears taken from various filamentous colonies. Note irregular staining in some of the longer filaments. Irregular shapes and forms can be seen in some of the smears (clubbed-shape, curved forms, curled forms, etc.).

was found that this sample brought better results in the primary plates. Subsequent transfers, however, survived better with the addition of small amounts of dextrose and $CaCl_2$.

DISCUSSION

In isolating these organisms some authors have claimed that the morphology as well as the requirement for oxygen had changed in later transfers.

Although the organisms required anaerobic conditions for the primary isolations, some of them were found to grow under aerobic conditions after a few transfers. The reports also showed that some of those that did grow appeared more like diptheroid than the filamentous forms that were originally isolated. There was some indication that smooth and rough variants occurred in this group of organisms and that this phenomenon could be demonstrated on agar plate cultures.

The filamentous forms isolated in this study remained anaerobic in their requirement and definitely maintained their filamentous morphology until they were lost. Many attempts were made to grow these forms under aerobic conditions but without avail. In working with these forms it can be seen that they grow very close to other organisms and that isolation becomes a very difficult procedure. Well isolated colonies can be obtained if plates are seeded rather carefully, and successful isolations of tiny colonies can be achieved by the use of a pasteur pipette of extremely fine diameter.

Washing of the soluble starch to remove the traces of reducing sugars and other water-soluble components appears to have aided in the growth of the filamentous forms indirectly. The other bacterial species did not grow as luxuriantly on these plates with the washed soluble starch, which might be an indication that some mouth organisms are capable of antagonizing or inhibiting the growth of the filamentous forms by the formation of harmful metabolic end products. Another possibility is that with the unwashed sample the other bacterial species were able to just mechanically overgrow the desired filamentous forms, since the over-all growth appeared more luxuriant on these plates.

The addition of small amounts of dextrose and $CaCl_2$ to the subsequent transfer medium has aided somewhat in the viability of these forms in view of the fact that they have been maintained through more transfers by the use of mediums supplemented with them. Perhaps the addition of other nutrients can further improve the qualities of this medium (TSY).

Since cultures have been kept for a period of eight transfers before being lost it would seem that the question of failure to supply needed nutrients would be ruled out for those kept for that length of time. There are, however, a few colonial types which have never been successfully transferred from the primary plates, and these are believed to require some additional nutrients for growth which were being supplied by one of the surrounding bacterial species on the primary plates. For those that were kept through a number of transfers, whatever nutrients that were carried over in the first transfer would soon dilute out through the succeeding transfers. This should approach the zero concentration by the third or fourth transfer, since isolated colonies were used in making the individual transfers in each case. From this point of view it would appear that the inability to continue to grow is perhaps because of some physical phenomenon or some other factor which is being overlooked.

Whether the organisms of the present study may be related or similar to other nonsporulating anaerobic rods, some of which at times appear as long filaments, is uncertain. There is not sufficient data concerning characteristics to justify a close comparison with other groups of bacteria. In general, the colony appearance, inability to grow in successive transfers, and lack of detectable odors in cultures are characteristics different from the majority of the anaerobic rods reviewed by Dack.[21]

SUMMARY

A simple medium has been presented for the isolation and cultivation of nonbranching filamentous mouth organisms. This medium, tryptose starch yeast extract agar, supplemented with a little dextrose and $CaCl_2$ has at times maintained these forms through eight transfers. The colonies of these forms can be readily identified by their morphology in spite of their small size. A few of the forms which have been isolated are presented, both in colony and microscopic morphology. They correspond closely to those observed by some of the earlier workers in this field. In spite of the fact that success has not yet been achieved in maintaining these forms indefinitely on laboratory mediums, this goal seems one step nearer since these forms have been isolated and carried in pure culture through several transfers.

Those cultures which have been maintained by the author over a period of tranfers remained anaerobic and all attempts to grow them under aerobic conditions failed. The cultures so maintained also remained filamentous, both in colonial and microscopic morphology.

The reason for failure to survive over a long period of time to permit a complete study of them has not as yet been ascertained. Cultures have been kept as long as the eighth transfer on several occasions with some being lost sooner. A few forms were observed which never could be isolated from the original plates.

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APYRASE IN PARTIALLY PURIFIED STAPHYLOCOCCAL ENTEROTOXIN

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In the course of studying partially purified enterotoxin for some characteristic biochemical property, an apyrase activity was found in the preparations. Although this enzyme does not appear to be enterotoxin, the methods so far used for the purification of the toxin[1,2] also concentrate the enzyme.

The phosphatases liberating orthophosphate from adenylphosphates have been isolated from muscle,[3,4] brain,[5] liver,[6] potato,[6,7] yeast,[8] Paramecium,[9] snake venoms,[10] and many other sources. Some of these enzymes are adenosinetriphosphatases and split only the terminal phosphate of adenosinetriphosphate; some are apyrases and liberate more than one phosphate grouping. The nucleotide pyrophosphatase of potato splits not only diphosphopyridine nucleotides but also adenylpyrophosphates.[11]

Studies of the apyrases of the true bacteria freed from the bacterial cells are limited. They have been obtained from a thermophile and *Bacillus subtilis* by the use of lysozyme[12] and from *Escherichia coli* by grinding with alumina.[13] However, the staphylococcal enzyme is found in the culture mediums in which the staphylococcus has been grown. The enzyme liberates inorganic phosphorus from adenosine triphosphate (ATP), adenosine diphosphate (ADP), adenosine-5-phosphate (AMP), and diphosphopyridine nucleotide (DPN).

MATERIALS AND METHODS

The partially purified enterotoxins and the materials obtained by similar purification procedures from non-enterotoxigenic strains of staphylococcus were used as the enzyme preparations;* no specific attempt was made to purify the enzyme per se. The phosphatase activities of the preparations derived from different strains of staphylococcus varied considerably. The data presented in this paper are concerned with two samples showing high activity. Preparation A had a high enterotoxic potency and preparation B (derived from a different strain of organism than that from which A was obtained) possessed very low enterotoxicity as determined by the monkey feeding test. Preparation A was purified from the crude enterotoxin by zinc precipitation, chromatography on alumina, and ethanol precipita-

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* Prepared by Dr. Surgalla and Dr. Bergdoll.

tion, yielding 1.94 mg of lyophilized material per original 100 ml of crude enterotoxin. Preparation B was obtained by successive treatment of the crude toxin by acid precipitation, chromatography on alumina, and ethanol precipitation, and represented 0.824 mg/100 ml of original crude toxin.[1,2]

ATP and ADP were sodium salts and were Pabst Laboratories products, as were the AMP and DPN.

The liberation of phosphate was determined indirectly by manometry (CO_2 production from a bicarbonate medium) or directly by the actual increase in inorganic phosphorus. The Warburg flasks contained (final volume of 3 ml) 0.047 *M* $NaHCO_3$, 0.028 *M* KCl, 0.004 *M* $MgCl_2$, 0.0165 *M* cysteine, and toxin with the substrate in the side arm. No Mg^{++} was used in experiments involving DPN. The cysteine, toxin, and substrate were dissolved and made to pH 8 just before use. The tests were run at 37 C after gassing with 4.7% CO_2+95.3% N_2 mixture and equilibrating a total of 30 minutes. The manometric method was less sensitive than the phosphorus determination method.

The reacting system for the direct method was the same as for the manometric method, except that 0.05 *M* glycyl-glycine buffer of pH 8.05 was substituted for the bicarbonate. All the reactants except the substrate were incubated together for 30 minutes at 37 C before the addition of the substrate. Mixing during the final incubation was kept to a minimum. After the desired incubation periods, an aliquot of the reacting mixture was cooled in ice water and pipetted into the 10 *N* H_2SO_4 used for the actual phosphorus determination. For the blanks the substrate and the remainder of the reactants were incubated separately, chilled in ice water, then mixed and pipetted into acid as rapidly as possible.

Inorganic phosphorus was determined by the method of Martin and Doty,[14] as modified by Ernster et al.[15] Trichloracetic acid gave incomplete precipitation of the materials present in the enzyme preparation; the use of silicotungstate and the isobutanol-benzene extraction overcame this difficulty. Since cysteine was necessary for the reaction, the concentration of $SnCl_2$ was increased 2½ times. The final concentration of cysteine was the same in all tubes and color readings were made immediately after the addition of the $SnCl_2$.

There was some variation in the data, especially in the direct method. This was probably due to errors in the weighing of the enzyme since only 1 to 2 mg amounts were weighed out for each experiment in order to conserve the suppy of the enzyme preparations. However, with the exception of the data of figure 4, each experiment was done with a single weighing of the enzyme so that the amount of enzyme in different tubes of a given experiment was constant.

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RESULTS

The enzymatic dephosphorylatin of ATP is dependent on the presence of cysteine. Reduced glutathione is not as effective an "activator" as is cystine (fig. 1) and ascorbic acid (manometric experiments) is ineffective. This preferential activation by cysteine of the staphylococcus enzyme is different from the brain phosphatase acting on ATP and inorganic pyrophosphate[5] and of native myosin acting on ATP,[16] where cysteine and glutathione seem to be equally effective. These results and the fact that the glycyl-glycine buffer increases the activity of the enzyme suggest the possibility that the cysteine is not acting as a reducing agent in the case of the staphylococcal enzyme but blocks some inhibitor present in the enzyme preparations.

The enzyme acting on ATP has a sharp optimum between pH 8.0 and 8.1. The presence of 0.028 *M* KCl resulted in greater activity, preparation A showing a relatively larger response to KCl than preparation B (fig. 2).

The enzymatic activity is not dependent on an exogenous source of cations. However, Mg^{++} and Co^{++} (0.004 *M*) and possibly Ca^{++} have some activating effect. On the other hand, the addition of the chelating agent disodium versenate (disodium salt of ethylene diamine tetraacetic acid) suppressed the ATPase reaction. Since the enzyme preparations are dialyzed against water,

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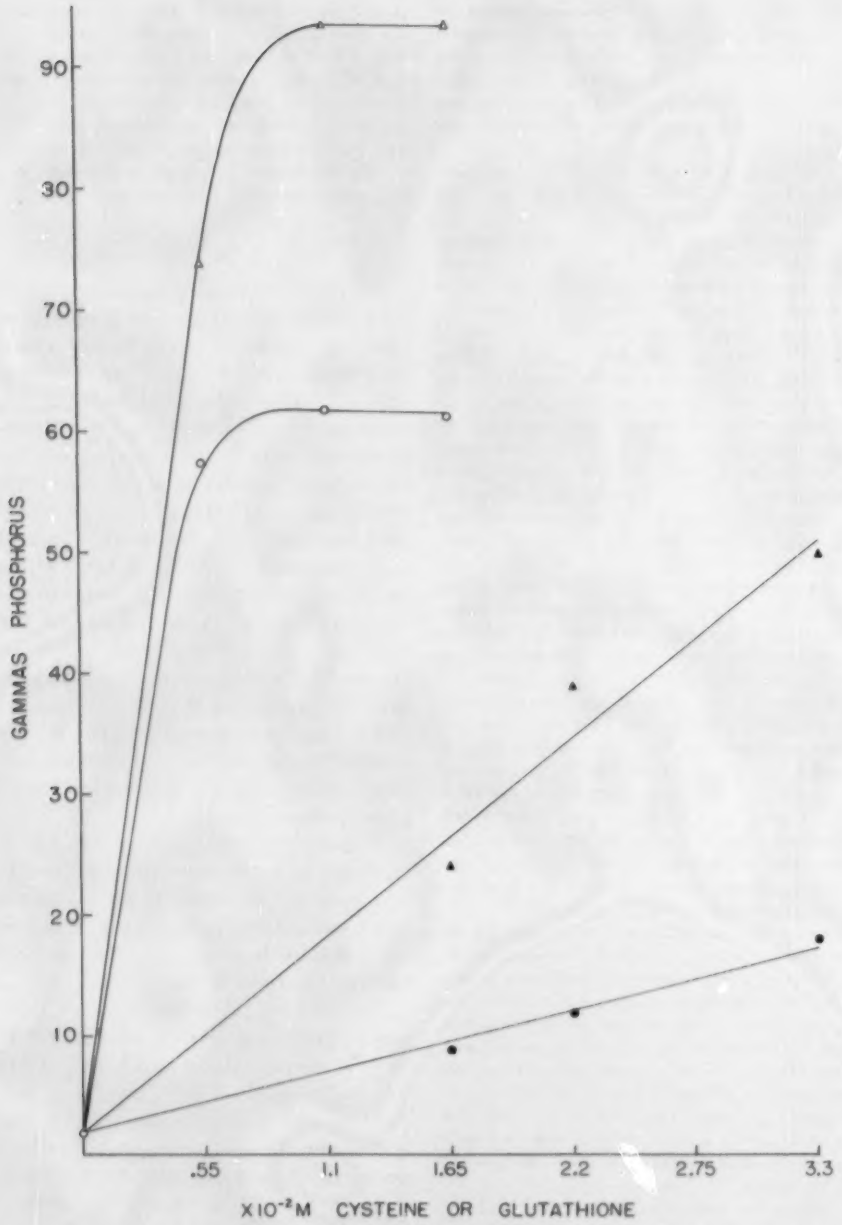

FIG. 1.—Activation of enzyme by cysteine and glutathione. μg P/30 min/3 ml reaction mixture. With 10 μmoles ATP and preparation A (0.1 mg): ●——● glutathione, ○——○ cysteine; preparation B (.08 mg): ▲——▲ glutathione, △——△ cysteine.

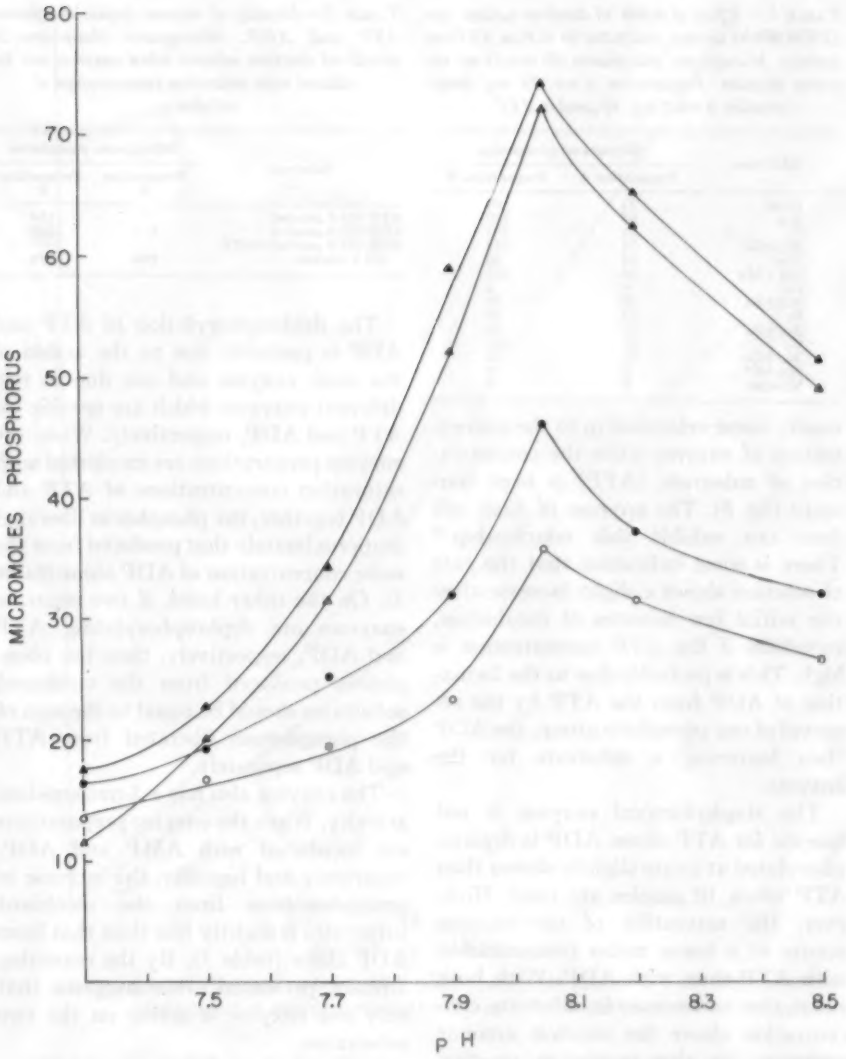

FIG. 2.—pH-activity relationship. μmoles P produced per mg enzyme per hr in 3 ml reaction mixture originally containing 10 μmoles ATP. KCl = 0.028 *M* when present. Preparation A: ○——○ without KCl, ●——● with KCl; preparation B: △——△ without KCl, ▲——▲ with KCl.

these results suggest that the cation may be bound in the enzyme preparation; 0.004 *M* Zn^{++} is inhibitory; the addition of 0.004 *M* Mg^{++} to a system containing the same concentration of Zn^{++} does not restore the original activity. Fluoride (0.002 *M*), even in the presence of 0.004 *M* Mg^{++}, inhibits the enzyme (table 1).

The velocity of reaction exhibits a

TABLE 1.—*Effect of 0.004 M divalent cations (as Cl^-), 0.004 M versene, and 0.002 M KF on ATPase activity. Micrograms phosphorus/30 min/3 ml reaction mixture. Preparation A = 0.125 mg, preparation B = 0.1 mg, 10 μmoles ATP.*

Additions	Micrograms phosphorus	
	Preparation A	Preparation B
None	55	99
Mg	87	110
Ca	67	102
Mg+Ca	74	115
Mn	55	88
Mg+Mn	65	115
Zn	9	4
Mg+Zn	20	29
Fe	53	60
Mg+Fe	57	86
Co	71	107
Mg+Co	61	89
$Mg+F^-$	0	8
Versene	0	0

nearly linear relationship to the concentration of enzyme when the concentration of substrate (ATP) is kept constant (fig. 3). The apyrase of *Esch. coli* does not exhibit this relationship.[18] There is some indication that the rate of reaction shows a slight increase after the initial few minutes of incubation, especially if the ATP concentration is high. This is probably due to the formation of ADP from the ATP by the removal of one phosphate group, the ADP then becoming a substrate for the enzyme.

The staphylococcal enzyme is not specific for ATP alone. ADP is dephosphorylated at a rate slightly slower than ATP when 10 μmoles are used. However, the saturation of the enzyme occurs at a lower molar concentration with ATP than with ADP. With both substrates an increase in substrate concentration above the smallest amount necessary to give maximum reaction velocity tends to suppress the reaction (fig. 4). Using the data of figure 4, the Qp values* are as follows: preparation A (9.85% N) with ATP = 1500 and with ADP = 3200; preparation B (13.89% N) with ATP = 2500 and with ADP = 4300.

* Qp = μl of H_3PO_4 split off per mg protein per hr at 38 C.[4] Values given represent tests run at 37 rather than 38 C.

TABLE 2.—*Identity of enzyme dephosphorylating ATP and ADP. Micrograms phosphorus/30 min/3 ml reaction mixture when enzymes are incubated with saturation concentration of substrates.*

Substrate	Micrograms phosphorus	
	Preparation A	Preparation B
ATP (12.5 μmoles)	74	133
ADP (25.0 μmoles)	173	233
ADP (25.0 μmoles) + ATP (12.5 μmoles)	166	228

The dephosphorylation of ATP and ADP is probably due to the action of the same enzyme and not due to two different enzymes which are specific for ATP and ADP, respectively. When the enzyme preparations are incubated with saturation concentrations of ATP and ADP together, the phosphorus liberated is approximately that produced from the same concentration of ADP alone (table 2). On the other hand, if two separate enzymes are dephosphorylating ATP and ADP, respectively, then the phosphorus produced from the combined substrates should be equal to the sum of the phosphorous liberated from ATP and ADP separately.

The enzyme also has a 5-nucleotidase activity. When the enzyme preparations are incubated with AMP and ADP, separately and together, the increase in orthophosphate from the combined substrates is slightly less than that from ADP alone (table 3). By the reasoning already presented, this suggests that only one enzyme is acting on the two substrates.

TABLE 3.—*Identity of enzyme acting on AMP and ADP. Micrograms phosphorus/30 min/3 ml reaction mixture when enzymes are incubated with AMP and ADP, separately and together.*

Substrate	Micrograms phosphorus	
	Preparation A	Preparation B
AMP (5 μmoles)	21	23
ADP (15 μmoles)	110	140
AMP (5 μmoles) + ADP (15 μmoles)	109	138

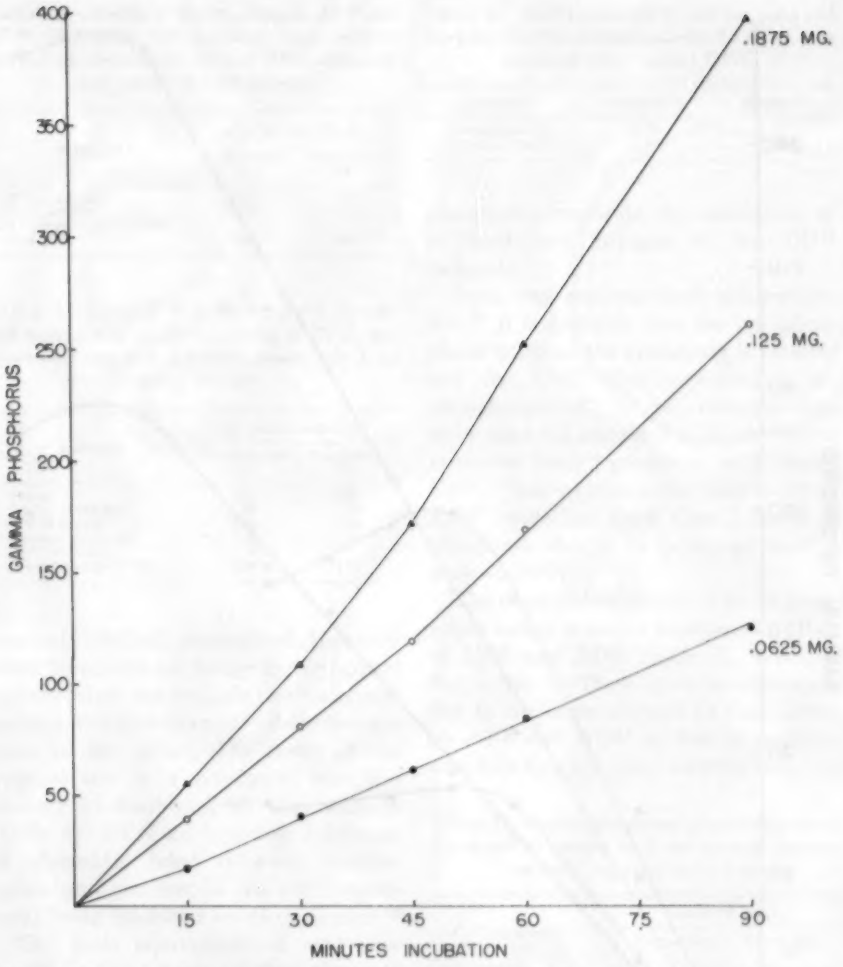

FIG. 3.—Reaction velocity with constant ATP concentration (10 μmoles in 3 ml) and increasing concentration of enzyme (preparation A). μg P/3 ml reaction mixture.

DPN is also a substrate for the enzyme dephosphorylating ATP, ADP, and AMP. Experiments analogous to that used to establish the identity of the enzyme acting on ATP and ADP are summarized in table 4.

The effect of the cations and fluoride ion on the degradation of DPN is different than when ATP is being dephosphorylated. KF, even at 0.028 *M*, is much less inhibitory and 0.004 *M* Mg^{++}, instead of enhancing the reaction, slows the action on DPN. Versene in 0.008 *M* concentration is an inhibitor (table 5).

This varying effect of metal ions and inhibitors when one enzyme acts on several substrates has also been shown for the potato nucleotide pyrophosphatase.[11]

The point of attack of the coenzyme molecule by the staphylococcal enzyme

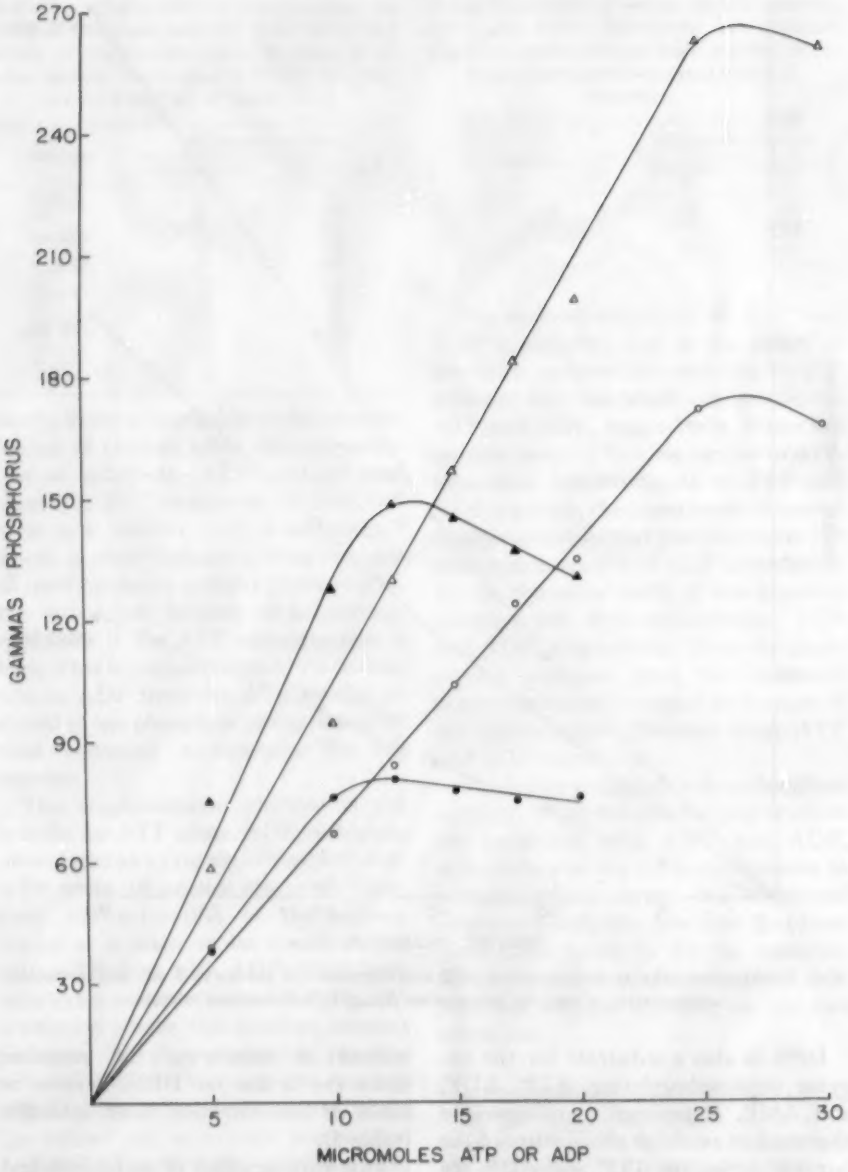

FIG. 4.—Effect of substrate concentration on reaction velocity with constant concentration of enzyme. μg P/30 min/3 ml reaction mixture. Preparation A (0.125 mg): ●——● with ATP, ○——○ with ADP; preparation B (0.1 mg): ▲——▲ with ATP, △——△ with ADP. Composite of three experiments.

TABLE 4.—*Identity of enzyme acting on ADP and DPN. Microliters CO_2 produced when enzymes (0.4 mg) incubated with ADP and DPN, separately and together in 3 ml volume.*

Substrate	μl carbon dioxide/hr	
	Preparation A	Preparation B
ADP (25 μmoles)	245	256
DPN (5 μmoles)	110	104
ADP (25 μmoles) + DPN (5 μmoles)	250	263

TABLE 5.—*Effect of KF, Mg^{++}, Zn^{++}, versene and nicotinamide on the breakdown of DPN. Enzymes = 0.5 mg and DPN = 5 μmoles per 3 ml reaction mixture.*

Additions	μl carbon dioxide/hr	
	Preparation A	Preparation B
None	132	133
Mg^{++} (0.004 *M*)	59	95
KF (0.028 *M*)	116	126
Versene (0.004 *M*)	0	19
Zn^{++} (0.004 *M*)	5	
Nicotinamide (0.1 *M*)	139	144

was not definitely determined. However since there was no change in the optical density when the cyanide test[17] was performed, the nicotinamide-ribose linkage must be left intact. The result of the cyanide test is in agreement with the absence of inhibition by nicotinamide (table 5), DPN nucleosidase (cleavage of glycosidic bond between nicotinamide and the rest of the DPN molecule) being inhibited by nicotinamide.[18]

The mole equivalents of acid produced (as CO_2) is greater than the mole equivalents of DPN used in the experiment (table 6). This indicates that the cleavage of DPN results in the formation of at least two new acid groups, one or all of which being weak and not completely dissociated at the pH of the experiment.[19] The liberation of inorganic

TABLE 6.—*Mole equivalents of acid (as CO_2) and inorganic phosphorus produced when 0.5 mg. enzyme incubated with 5 μmoles DPN.*

Enzyme	μmoles CO_2	μmoles P
Preparation A	5.89	5.20
Preparation B	5.94	4.85

phosphorus confirms the disruption of at least two linkages of the DPN molecule.

Since the enzyme dephosphorylates AMP, it is probable that the pyrophosphate bond of the coenzyme is cleaved and the AMP thus produced is dephosphorylated. In no instance was more than 5.2 μmoles of orthophosphate produced from 5 μmoles of 95% purity DPN. This excludes the possibility of ADP formation, since then 2 moles of phosphate should be produced from 1 mole of DPN.

The dephosphorylation of other phosphate esters is minor compared to that of ATP and ADP (table 7). Whether the action on these other substrates is due to the same enzyme as that acting on ATP and ADP or due to separate enzymes has not been ascertained.

TABLE 7.—*Dephosphorylation of phosphate esters. Substrates 10 μmoles in 3 ml reaction mixture. μmoles P/mg toxin/hr in 3 ml.*

Substrate	μmoles P/mg/hr	
	Preparation A	Preparation B
Adenosine triophosphate	47.0	74.5
Adenosine-3-phosphate	5.6	1.2
Sodium pyrophosphate	0	3.3
Sodium-β-glycerophosphate	2.3	0
Glucose-1-phosphate	0.4	4.3
Fructose-1-6-phosphate	0.4	0.2
Phenyl disodium phosphate	0	0

DISCUSSION

In view of the almost universal distribution of ATPase and apyrase type enzymes in living cells, the finding of an apyrase type of enzyme of staphylococcus is not surprising. Hitherto, cell-free bacterial apyrase have been ob-

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19. Chain, E. 1939, Biochem. J. **33**: 407–411.

tained by disrupting the cells with lysozyme[12] or by grinding with alumina.[13] The staphylococcal enzyme has been concentrated from the culture medium in which the organism has been grown after removal of the cells by centrifugation. Whether this is due to the liberation of the enzyme by autolysis or by secretion is unknown. The enzymatic activity seemed higher when the partially purified enterotoxin which contains the enzyme was processed from a large volume of growth fluid than from a small volume.

Although the methods used so far in the purification of enterotoxin also concentrate the enzyme, the apyrase does not seem to be enterotoxin. There is no correlation between the enzymatic activity and the enterotoxigenicity. Moreover, the apyrases of the preparations derived from enterotoxigenic and nonenterotoxigenic strains show no essential differences in the properties studied.

In the detection of enterotoxin parenteral introduction of the material into test animals is sometimes used.[20] The presence of the apyrase activity in these enterotoxin preparations would seem to be another reason for avoiding this method, unless the apyrase can be specifically inactivated or removed without affecting the enterotoxin. The intravenous injections of some of the available enterotoxin preparations into cats have produced shock-like syndrome and even death.[21] This toxicity may possibly be due to the apyrase since some of the symptoms of snake venom poisoning have been ascribed to the enzymes breaking down adenylphosphates and DPN.[22]

The gel-diffusion precipitin technic shows the presence of a number of antigens in the enterotoxin preparations[23,24]; this indicates that the preparations have contaminating substances. One of the antigens is probably the apyrase, since the enzymatic activity can be neutralized with immune serum[21]. Some of the other antigens probably account for the effect of the enterotoxin preparations on several other enzymatic and pharmacological test systems[21]; however, none of these effects can be ascribed to the enterotoxin itself.

SUMMARY

An apyrase type enzyme of staphylococcus has been shown to liberate inorganic phosphorus from adenosine triphosphate, adenosine diphosphate, adenosine-5-phosphate, and diphosphopyridine nucleotide. The enzyme is concentrated by the methods being used in the purification of enterotoxin but is not the enterotoxin itself.

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22. Zeller, E. A. 1951, The Enzymes, edited by J. B. Sumner and K. Myrback, Academic Press.
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24. Surgalla, M. J., Bergdoll, M. S. and Dack, G. M. 1954, J. Immunol. 72: 398–403.

THE INDEX

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VOLUME 56 MAY-JUNE 1935 NUMBER 3

The Journal of Infectious Diseases

TABLE OF CONTENTS

PAGE

WILLIAM H. TALIAFERRO AND TULIO PIZZI. Connective tissue reactions in normal and immunized mice to a reticulotropic strain of *Trypanosoma cruzi*. 199

W. J. NUNGESTER, VINCENT MOORE, LEONARD MIKA, AND PERRY WILLIAMS SUMMERS. Comparative study of host resistance of guinea pigs and rats. I. Virulence of *Diplococcus pneumoniae* and *Bacillus anthracis* for guinea pigs and rats. 227

MILES E. HENCH AND W. J. NUNGESTER. Comparative study of host resistance of guinea pigs and rats. II. Effect of guinea pig and rat serum on *Diplococcus pneumoniae* type I. 232

BERNARD N. JASINSKY. The effect of X irradiation on immunity of the mouse to *Trypanosoma duttoni*. 242

GLEN R. GALE. The effects of β-propiolactone on the metabolism of *Candida albicans*. 250

WILLIAM CANTRELL. The effects of cortisone and oxophenarsine on *Trypanosoma equiperdum* infections in the rat. 259

ELLEN M. SIMPSON, M. SCOTT REDPEARTH AND DAVID T. BERMAN. Competitive values of smooth and rough *Brucella abortus* in mixed infections in mice. 268

GEORGE J. KARAS. A study of mediums for the growth and isolation of filamentous mouth organisms. 279

[illegible] SUGIYAMA AND G. M. DACK. Apyrase in partially purified staphylococcal enterotoxin. 286

[illegible] 295